REMARKS

Summary of Interview

As an initial matter, applicant wishes to expresses his gratitude to the Examiner for the courtesies extended to Applicants' undersigned representatives during the telephonic interview conducted on June 4, 2007. In that interview, claim 51 was discussed. The interview did not concern patentability of the claims, but rather, procedural issues related to an earlier issued restriction requirement. Specifically, applicants' representatives inquired as to whether adding "for the treatment of atherosclerosis" to independent claim 51 would render the reply by applicants unresponsive due to the restriction requirement. The Examiners advised applicant's representatives as to the possible forms of such an amendment and possible consequences thereof. Further, the issue of priority with respect to Xiao, et al., and Kapeller-Libermann, et al., was briefly discussed. No specific conclusions or agreement was reached regarding any of the above.

Status of Claims and Review

Claims 51-61 remain under active prosecution in the present application. Claims 37-50 and 62-65 are withdrawn as being drawn to a non-elected invention. Claims 1-36 have been previously canceled. Claims 51, 54 and 56 are currently amended. Applicants respectfully submit that all amendments are supported by the original disclosure and do not introduce new matter.

By way of review, the pending claims relate to methods of providing biologically active lipid hydrolyzing proteins or polypeptides to cells of a mammal comprising administering into cells a vector that comprises a DNA sequence encoding the lipid hydrolyzing protein. One such lipid hydrolyzing protein that may be used with the claimed methods is lysosomal acid lipase (LAL). Such proteins are useful for the treatment and prevention of atherosclerosis.

In the subject Office Action dated March 7, 2007, the Office has rejected claims 51-61 under 35 U.S.C. §112, first and second paragraph, and claims 51-54 and 56-61 under 35 U.S.C. §102. Applicants' arguments in response to these rejections are set forth below.

Amendments to Claims 51, 54 and 56

In the interests of advancing prosecution and more clearly pointing out applicants' invention, claims 51, 54 and 56 have been amended as follows:

In claim 51, the language "having a deficiency in biologically active lipid hydrolyzing protein or polypeptide" has been deleted to more accurately reflect the scope of the disclosure, in that cells which *do not* have a deficiency in biologically active lipid hydrolyzing protein may be provided additional protein using the claimed methods to achieve a desired result. Support for this amendment may be found at ¶¶ [0031] and [0032]. Claim 51 has also been amended to add the feature of sequence containing "the catalytic lipase triad Asp-Ser-His" support for which may be found at ¶¶ [0034] and [0035] to more clearly define the scope of the claim.

Claim 54, as is discussed below, has been amended to more clearly point out the invention by including the feature of "showing biological activity similar to that of lysosomal acid lipase." Support for this amendment may be found, for example, in original claim 7.

Claim 56 has been amended in a manner similar to that of claim 51. In claim 56, the language "having a deficiency in biologically active lysosomal acid lipase" has been deleted to more accurately reflect the scope of the disclosure, in that cells which do not have a deficiency in biologically active lipid hydrolyzing protein may be provided additional protein using the methods to achieve a desired result. Support for this amendment may be found at ¶¶ [0031] and [0032].

Claim Rejection - 35 USC § 112, second paragraph

The Office has rejected claims 54 and 55 under 35 USC §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. The Office bases this rejection on the fact that no polypeptide

sequence of any lysosomal acid lipase is disclosed in the specification, rendering the phrase "substitution of amino acid Pro(-6) to Thr and Gly2 to Arg" vague and indefinite. Applicants are unclear why claim 54 is rejected on this basis, as no explanation is provided in the Office Action. However, applicants remarks to the rejection to the language of claim 55 is set forth below.

The Office is reminded that a specification need not disclose, and preferably omits, that which is well-known to one of skill in the art. MPEP 2164.05(a), citing *In re Buchner*, 929 F.2d 660, 661, 18 USPQ 2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), cert. denied, 480 U.S. 947 (1987); and *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 USPQ 481, 489 (Fed. Cir. 1984). With respect to the lysosomal acid lipase sequence, both the amino acid and DNA sequence were published and well-known in the art at the time of filing. In particular, Ameis et al, *Eur. J. Biochem.* 1994, referenced by applicants at paragraph [0037] discloses the cDNA and peptide sequence sufficient to enable the instant invention. With respect to claim 55, the nomenclature used would readily be understood to one of skill in the art at the time of filing. To clarify, "Gly2 to Arg" indicates that the naturally-occurring amino acid at position 2 in lysosomal acid lipase is mutated from glycine to arginine. Likewise, "Pro(-6) to Thr" indicates the proline residue six amino acids upstream of the first amino acid of the lysosomal acid lipase protein is mutated to a threonine. As such, applicants' respectfully assert that the claim is not vague or indefinite, and the rejection to the claim should be withdrawn.

To the extent claim 54 was rejected under 35 U.S.C. §112, second paragraph, for failing to disclose the polypeptide sequence of any lysosomal acid lipase, the above arguments apply. Both the polypepide and cDNA sequence (from which a peptide sequence could readily be determined) are disclosed in the Ameis reference cited by the applicants. As such, the claims, as directed to lysosomal acid lipase, do, in fact, particularly point out and distinctly claim the invention.

For at least the above reasons, Applicants respectfully submit that the objections to claims 54 and 55 as indefinite under 35 U.S.C. §112, second paragraph, are overcome and should be withdrawn.

Claim Rejection - 35 USC § 112, first paragraph

The Office has rejected claims 51-61 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, that the inventor had possession of the claimed invention.

The Office has rejected claims 51-61 on this basis, asserting that the applicants were not in possession of the genus of lysosomal acid lipase cDNAs or the broader genus of lipid hydrolyzing protein DNAs. Office Action, p. 6. The Office bases this rejection on, in part, the conclusion that lysosomal acid lipases, variants, and fragments thereof have not been disclosed, and that there "is no evidence on the record of a relationship between the structure of any lysosomal acid lipase cDNA and the claimed lysosomal acid lipase DNA that would provide any reliable information about the structure of other lysosomal acid lipase DNAs within the genus." Office action at pp. 5-6. Further, the Office asserts that, because applicants are not in possession of the narrower genus of "lysosomal acid lipase cDNAs" that applicants further cannot be in possession of the broader class of "lipid hydrolyzing protein cDNAs." Applicants respectfully disagree, and believe that the record adequately demonstrates possession of the claimed invention.

The Office has rejected claim 51, asserting that there "is no evidence that the asserted lipid hydrolyzing protein cDNA had a known structural relationship to any other lipid hydrolyzing protein cDNA sequence." Office Action at p.5. Applicants respectfully disagree. In an effort to advance prosecution and more clearly define the claimed invention, applicants have amended claim 51 to more clearly define the structural relationship to other lipid hydrolyzing protein cDNA. Claim 51 now claims lipid hydrolyzing protein sequences which contain the catalytic lipase triad Asp-Ser-His, where the Ser is a Ser^{153} residue." This amendment to the claims demonstrates a specific structural relationship to other lipid hydrolyzing proteins. As such, one of ordinary skill in the art would recognize other proteins or polypeptides (i.e., those containing the catalytic lipase triad of amino acids) which could be used with the instant invention. As such, the rejection to claim 51 and claims dependent thereon is overcome and should be withdrawn.

The Office has also rejected those claims directed towards lysosomal acid lipases (claims 56-61) as lacking sufficient structure that would provide any reliable information about the structure of other lysosomal acid lipase DNAs. Office Action, p. 5. Applicant respectfully disagrees. As set forth above, the lysosomal acid lipase protein was well-characterized at the time the instant application was filed. In particular, as noted by applicants at paragraph [0037], conserved regions of lysosomal acid lipase were known and published prior to filing of the instant application. In general, the level of skill in the art is such that knowledge of conserved regions in a protein allows one to predict the general structure of operable variants such that every variant need not be disclosed. Further, at the time of filing, a functional assay was available which could be used to identify active lipid hydrolyzing proteins, particularly LAL. See Sheriff, et al., *J. Biol. Chem.* 1995; 270:27766-27772, a copy of which is enclosed. As such, applicants respectfully submit that applicants were, in fact, in possession of the genus of lysosomal acid lipase DNAs, and that one of skill in the art could readily ascertain biologically active LAL or lipid hydrolyzing proteins or polypeptides. The rejection as applied to claims 56-61 should therefore be withdrawn, the claims now being in condition for allowance.

The Office has also rejected claim 54 in view of the phrase "at least 85% homology." Office Action, p. 6. The Office asserts that the claim is not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed invention, as there is no basis for determining homology. Claim 54 has been amended to clarify the claim and to advance prosecution. Claim 54 now refers to sequences or polypeptides having at least 85% sequence homology to lysosomal acid lipase showing biological activity similar to that of lysosomal acid lipase. Support for this amendment can be found throughout the application, for example, in original claim 7.

In view of this amendment, applicant believes this amendment limits the number of possible variants such that it is now clear that only a finite and determinable number of variants can be made such that the protein or polypeptide having at least 85% sequence homology has similar biological activity to lysosomal acid lipase. One of ordinary skill in the art will understand that a limited number of substitutions or alterations could be made in a protein or polypeptide while still possessing the requisite biological activity. The number of *possible* polypeptides is not a relevant inquiry, provided that operable polypeptides are sufficiently

predictable. Given the high level of skill in the art and the guidance provided by the specification, such variants can readily be determined by one of ordinary skill in the art without undue experimentation using methods known at the time of filing. As such, applicants respectfully submit that for at least the above reasons, claim 54 now more clearly complies with 35 U.S.C. §112, second paragraph, and is in form for allowance.

35 U.S.C. §112, first paragraph

The Office has rejected claims 51-61 under 35 U.S.C. §112, first paragraph, as failing to comply with the enablement requirement, containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention.

The Office is respectfully reminded that the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. See MPEP 2164, citing *In re Certain Limited-Charge Cell Culture Microcarriers* (Int'l Trade Comm'n 1983). Applicants respectfully assert that such is the case at present. Further, it is not necessary to "enable one of ordinary skill in the art to make and use a perfected, commercially viable embodiment absent a claim limitation to that effect." See MPEP 2164, citing *CFMT, Inc. v. Yieldup Int'l Corp* (Fed. Cir. 2003). The instant claims lack such a limitation. Finally, "[d]etailed procedures for making and using the invention may not be necessary if the description of the invention itself is sufficient to permit those skilled in the art to make and use the invention." MPEP 2164. Applicants respectfully that such is the case with respect to the instant claims.

The Wands Factors

As set forth by the Office, enablement is considered in view of the *Wands* factors MPEP 2164.01(a). Under *Wands*, enablement is not precluded by the necessity for experimentation such as routine screening, but practice of the invention must not require *undue* experimentation. The factors to be considered in determining whether undue experimentation is required include:

(1) the quantity of experimentation necessary, (2) the amount or direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. These factors are discussed in turn with respect to the instant invention.

The nature of the invention: The nature of the invention is a method of administering a polynucleotide sequence encoding a lipid hydrolyzing protein, such as the well-characterized lysosomal acid lipase protein (LAL), to cells using a vector to provide biologically active protein or polypeptide in the cell.

The breadth of the claims: The breadth of the invention encompasses the administration of a lipid hydrolyzing protein or peptide sequence to increase levels of this protein in cells. The claims are limited in scope to administration of lipid hydrolyzing proteins and lipid hydrolyzing proteins possessing similar biological activity to lysosomal acid lipase. While a variety of different methods may be used to administer the DNA, the claims are limited to this specific class of DNA sequences having a specific biological activity.

The state of the prior art: The Office has cited Wolf, et al., summarizing the molecular mechanisms of mobilization of fat from adipose tissues, published in 2005. Applicant is unclear as to why this reference (published in 2005) was cited. The Office is invited to contact the representatives of the applicant if an explanation is indicated.

With respect to the remainder of this section in the Office Action, the Office has cited several references describing the state of gene therapy. Firstly, the Office cites Pouton et al. for the proposition that "direct injection of gene medicine into target tissue represents a far simpler task than targeting delivery to a specific tissue from the systemic circulation." Office Action, p. 10. However, a statement that a particular method is more difficult does not mean that the method requires undue experimentation, which is the applicable standard. Johnson-Saliba is cited for the proposition that in clinical trials, "true success has been limited" and Read et al. is cited for the proposition that the "lack of suitable vectors for the delivery of nucleic acids... represents a major hurdle to their continued development and therapeutic application." However, these references merely suggest the difficulties inherent in gene therapy methods, and such discussion does not

necessarily render experimentation undue. Challenges and difficulties in practicing an invention, however, does *not* necessarily equate to *undue* experimentation.

In fact, applicants respectfully submit that the references offered by the Office actually *support* a finding that gene therapy techniques are within the skill of the art at the time of filing, such that undue experimentation is not required. In Read, et al., at p. 21, for example, the authors note that administration of DNA/lipid mixtures is safe and can produce clinically significant responses. Further, hydrodynamic delivery to the liver via a superficial vein is referred to as the simplest successful in vivo delivery route, having few barriers to delivery. (Read, et al., p.22).

To further elucidate the high level of skill in the art at the time of filing, the following references are provided. At the time of filing, successful administration of genes to produce biologically active proteins was known and described. The following references, which exemplary, are enclosed:

- Rosengart, T, "Angiogenesis Gene Therapy: Phase I Assessment of Direct Intramyocardial Administration of an Adenovirus Vector Expressing VEGF121 cDNA to Individuals with Clinically Significant Severe Coronary Artery Disease," *Circulation* 1999; 100:468-474, "Rosengart I"; and Rosengart, T., et al., "Six-Month Assessment of a Phase I Trial of Angiogenic Gene Therapy for the Treatment of Coronary Artery Disease Using Direct Intramyocardial Administration of an Adenovirus Vector Expressing the VEGF121 cDNA," *Annals of Surgery* 1999; 230: 455-472, "Rosengart II."
- Losordo, D.W., et al, "Gene Therapy for Myocardial Angiogenesis: Initial Clinical Results with Direct Myocardial Injection of phVEGF165 as Sole Therapy for Myocardial Ischemia," *Circulation* 1998; 98:2800-2804.
- Shetty, K. et al, "Gene therapy of hepatic diseases: prospects for the new millennium," *Gut* 2000; 46:136-139
- Hirschowitz, E., "Regional treatment of hepatic micrometastasis by adenovirus vector-mediated delivery of interleukin-1 and interleukin-12 cDNAs to the hepatic parenchyma," *Cancer Gene Therapy* 1999; 6:491-498.

In Rosengart I and II, both published in 1999, a recombinant adenovirus (Ad) gene transfer vector containing vascular endothelial growth factor (VEGF) cDNA was successfully administered directly to an ischemic area of the myocardium in patients with coronary artery disease, showing cardiovascular improvement both one and six months after treatment with the gene. Combined, these references demonstrate the feasibility and successful application of vector-mediated gene delivery, showing beneficial effects without apparent toxicity for up to six months after administration of the vector. In the discussion following the publication (see p.470, bottom of page), Dr. Judah Folkman notes that the procedure is safe, with few if any side effects, and that myocardial function is improved. This reference demonstrates the level of skill in the art and the successful application of gene therapy methods to deliver active proteins *at the time of filing the instant application.*

Similarly, Losordo et al. demonstrates successful gene transfer of DNA (encoding VEGF) to the myocardium. Losordo showed no operative complications, and marked symptomatic improvement and/or objective evidence of improved myocardial perfusion in all patients. This data shows that administration of a protein using a vector containing the DNA of a protein could be successfully administered and expressed at the time of filing the instant application.

Further, Shetty et al. describe, for example, successful methods of gene delivery to the liver, including retroviral vectors, adenoviral vectors, adeno-associated vectors, simian virus 40 vectors, and hybrid viruses. Shetty additionally sets forth non-viral methods of gene delivery using, for example, conjugates or liposomes known at the time of filing.

As a final example, Hirschowitz et al. demonstrate successful use of adenovirus vector-mediated delivery of DNA sequences to produce high levels of corresponding protein in the liver. As such, this reference is a yet further example of the high level of skill in the art at the time of filing, such that administration of DNA to produce biologically active protein could be successfully practiced.

In summary, the above references—which are but a small number of references available to support the feasibility of gene therapy methods at the time of filing— individually and collectively, demonstrate the feasibility of administering DNA sequence to cells resulting in

expression of biologically active protein. As such, these references demonstrate the high level of skill in the art such that undue experimentation would not be required to practice the claimed invention.

The predictability or lack thereof in the art: There is, in fact, some degree of predictability with respect to the administration of DNA to provide biologically active protein in vivo, as evidenced by the above cited references. In fact, as demonstrated by the references cited above, the art is sufficiently predictable to allow successful use of gene therapy methods. Applicants respectfully submit that the above cited references demonstrate that DNA delivery methods at the time of filing were sufficiently developed such that practice of the claimed invention would not require undue experimentation.

The amount of direction or guidance: The specification of the instant application discloses a novel method of treating diseases in which cholesteryl esters and triglycerides accumulate. This method comprises administering DNA sequences encoding lipase hydrolyzing or LAL-like proteins. As noted by the Office, vectors expressing proteins were disclosed. In combination with methods known in the art at the time of filing, such as those described in the articles cited above, the claims of the instant invention could be carried out without undue experimentation.

The Office has further stated that the phrase "at least 85% sequence homology to lysosomal acid lipase" recited in claim 54 is not enabled, stating that one skilled in the art could not make and use the claimed invention. In view of the amendments made to claim 54, requiring that the sequence having at least 85% homology have similar biological activity, and those arguments set forth on page 10, applicants respectfully assert that practice of the invention with respect to this claim feature would merely require routine experimentation.

In view of the above, particularly the high level of skill in the art and the successful practice of gene therapy at the time of filing, applicants believe that undue experimentation is not required to practice the claimed invention. Accordingly, for at least all of the above reasons, favorable reconsideration and withdrawal of the rejection of claims 51-61 under 35 U.S.C. § 112 are respectfully requested.

In the event that the Office maintains this enablement rejection, Applicant respectfully requests, in accordance with the principles of compact prosecution, that the Office articulate, on the record and with specificity sufficient to support a prima facie case of non-enablement, the factual basis on which it is alleged that it would be beyond the level of ordinary skill in the widget art to make and use the claimed invention without undue experimentation. MPEP §2164.01.

Claim Rejections - 35 USC §102

The Office Action cites Kapeller-Libermann and Xiao against the instant claims under 35 U.S.C. 102(e). Assuming that the application is entitled to the benefit of the earlier filed provisional applications, the earliest possible 102(e) dates for the Kapeller-Libermann reference is January 25, 2001. The Xiao reference does not claim priority to any earlier filed applications, and as such, the 102(e) date for Xiao is the filing date, which is October 26, 2001. Even if Xiao claimed priority to an earlier filed application during prosecution subsequent to publication, the earliest possible priority date would be October 31, 2000. The instant invention is entitled to a priority of invention date which is earlier than October 31, 2000 for at least the reasons described below and in the attached declaration. As such, these references cannot be prior art to the instant invention and the rejections under 102(e) are rendered moot and should be withdrawn.

1. The instant application is entitled to the benefit of the earlier-filed provisional application, which has a filing date of April 4, 2000.

The instant application is entitled to the priority date of the provisional application (US 60,180,362) filed on February 4, 2000 because the provisional application disclosed endogenous methods of protein delivery. The Office has declined applicants the benefit of the earlier-filed provisional application, stating that the provisional did not disclose administration of DNA sequences, and thus cannot be the basis for priority. The Office has therefore limited priority to the filing date of the parent application, February 2, 2001. Applicant respectfully disagrees with this conclusion.

The provisional application evidences that the inventors were in possession of the claimed invention, entitling the inventors to claim the benefit of this filing date. The Office is referred to page 3, lines 10-15 of provisional application US 60,180,362, wherein the

endogenous method of delivery is disclosed. The provisional application states that the endogenous protein is "produced or manufactured inside the body by some type of device (biologic or other) for delivery to within or to other organs of the body." This encompasses delivery of DNA for production of proteins by cells endogenously. As such, the provisional application provides support for the pending claims, and accordingly, the priority date of the instant application should be the filing date of this provisional, February 4, 2000, which predates both Kapeller-Libermann and Xiao.

2. Applicants herewith submit facts in support of invention prior to the 102(e) dates of both Kapeller-Libermann and Xiao.

As set forth above, applicants need only demonstrate a priority date before October 31, 2000. In support of a priority date before January 25, 2001, applicants submit the attached declaration under 37 C.F.R. § 1.131 and Exhibits which set forth facts that evidence priority of invention. In view of this evidence, the cited references are not prior art against the pending claims.

For at least the above reasons, the rejection under 35 U.S.C. 102(e) has been overcome and should be withdrawn.

CONCLUSION

In summary, the rejections under 35 USC §112, first and second paragraph, and 35 USC §102(e) have been overcome and should be withdrawn.

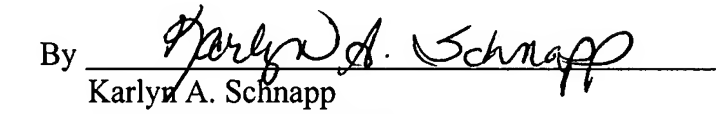
It is therefore respectfully submitted that the claims currently pending in the present application are in form for allowance. Accordingly, reconsideration of those claims, as amended herein, is earnestly solicited.

Applicants also make note herein that the absence of additional patentability arguments should not be construed as either a disclaimer of such arguments or an indication that applicants believe that such arguments are not meritorious.

The Commissioner for Patents is hereby authorized to charge any deficiency or credit any overpayment of fees to Frost Brown Todd LLC Deposit Account No. 06-2226.

Respectfully submitted,

GREGORY A. GRABOWSKI, et al.

By 

Karlyn A. Schnapp
Registration No. 45,558
Attorney for Applicant(s)
FROST BROWN TODD LLC
2200 PNC Center
201 East Fifth Street
Cincinnati, Ohio 45202
(513) 651-6865
salbainyjenei@fbtlaw.com

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with EXPRESS MAIL to be filed with the Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, this 7th day of August, 2007.

Randall K. Gaunce

Randall K. Gaunce

Angiogenesis Gene Therapy

Phase I Assessment of Direct Intramyocardial Administration of an Adenovirus Vector Expressing VEGF121 cDNA to Individuals With Clinically Significant Severe Coronary Artery Disease

Todd K. Rosengart, MD; Leonard Y. Lee, MD; Shailen R. Patel, MD; Timothy A. Sanborn, MD; Manish Parikh, MD; Geoffrey W. Bergman, MBBS; Rory Hachamovitch, MD; Massimiliano Szulc, PhD; Paul D. Kligfield, MD; Peter M. Okin, MD; Rebecca T. Hahn, MD; Richard B. Devereux, MD; Martin R. Post, MD; Neil R. Hackett, PhD; Taliba Foster, MS; Tina M. Grasso, BS; Martin L. Lesser, PhD; O. Wayne Isom, MD; Ronald G. Crystal, MD

Background—Therapeutic angiogenesis, a new experimental strategy for the treatment of vascular insufficiency, uses the administration of mediators known to induce vascular development in embryogenesis to induce neovascularization of ischemic adult tissues. This report summarizes a phase I clinical experience with a gene-therapy strategy that used an $E1^-E3^-$ adenovirus (Ad).gene-transfer vector expressing human vascular endothelial growth factor (VEGF) 121 cDNA (Ad_{GV}VEGF121.10) to induce therapeutic angiogenesis in the myocardium of individuals with clinically significant coronary artery disease.

Methods and Results—Ad_{GV}VEGF121.10 was administered to 21 individuals by direct myocardial injection into an area of reversible ischemia either as an adjunct to conventional coronary artery bypass grafting (group A, n=15) or as sole therapy via a minithoracotomy (group B, n=6). There was no evidence of systemic or cardiac-related adverse events related to vector administration. In both groups, coronary angiography and stress sestamibi scan assessment of wall motion 30 days after therapy suggested improvement in the area of vector administration. All patients reported improvement in angina class after therapy. In group B, in which gene transfer was the only therapy, treadmill exercise assessment suggested improvement in most individuals.

Conclusions—The data are consistent with the concept that direct myocardial administration of Ad_{GV}VEGF121.10 to individuals with clinically significant coronary artery disease appears to be well tolerated, and initiation of phase II evaluation of this therapy is warranted. (***Circulation.*** **1999;100:468-474.)**

Key Words: angiogenesis ■ gene therapy ■ genetics ■ coronary disease ■ ischemia

A new experimental strategy for treating myocardial ischemia is to induce neovascularization of the heart by use of "angiogens," mediators that induce the formation of blood vessels.[1,2] This approach is based on the knowledge that in the adult heart, the genes coding for angiogens and their receptors are expressed in low levels, apparently insufficient in most individuals to provide robust formation of collaterals in response to chronic ischemia.[3,4]

Vascular endothelial growth factor (VEGF), a protein coded by a 7-exon gene localized on chromosome 6, serves as a major angiogen in normal cardiac development.[5] The *VEGF* gene is normally spliced into 4 different forms; of these, VEGF121 (containing 121 amino acids) and VEGF165 (165 amino acids) appear to be the most important. The VEGF proteins function by interacting with specific receptors on endothelial cells, which initiates a cascade of events culminating in endothelial cell migration, proliferation, aggregation into tubelike structures, and networking of the arterial and venous systems.[2,5–8]

Gene transfer represents one approach to delivering an angiogen to the heart in which the cDNA coding for VEGF is delivered to the myocardium, with the myocardial cells used to secrete the VEGF.[8–10] Studies in experimental animals have shown that replication-deficient, recombinant adenovi-

Received February 8, 1999; revision received May 9, 1999; accepted May 13, 1999.

From the Department of Cardiothoracic Surgery (T.K.R., L.Y.L., S.R.P., O.W.I.), Division of Pulmonary and Critical Care Medicine (L.Y.L., S.R.P., N.R.H., T.F., R.G.C.), Division of Cardiology (T.A.S., M.P., G.W.B., R.H., M.S., P.D.K., P.M.O., R.T.H., R.B.D., M.R.P.), Weill Medical College of Cornell University–New York Presbyterian Hospital, New York, NY; GenVec, Inc (T.M.G.), Rockville, Md; and Division of Biostatistics (M.L.L.), North Shore University Hospital, Manhasset, NY.

Dr Rosengart is a consultant to GenVec, Inc, a privately held biotechnology company that is engaged in the business of designing and manufacturing vectors. Dr Crystal has equity in and is a consultant to GenVec, Inc.

Correspondence to R.G. Crystal, MD, Weill Medical College of Cornell University, New York Presbyterian Hospital, New York, NY 10021. E-mail geneticmedicine@mail.med.cornell.edu

Circulation is available at http://www.circulationaha.org

TABLE 1. Demographics and Intraoperative and Postoperative Characteristics of the Study Population

Parameter	Group A Adjunct to CABG	Group B Sole Therapy/ Minithoracotomy
n	15	6
Male/female, n	11/4	4/2
Age, y	60±10 (45–83)	59±11 (40–73)
Ejection fraction, %*	45±10 (28–63)	34±12 (20–50)
Prior MI, %	60	83
Diabetes, %	60	50
Hypertension, %	93	67
History of CHF, %	27	33
Prior CABG, %	27	83
Prior PTCA, %	13	33
Hypercholesterolemia, %	27	67
CABG procedure,† %		NA
×3	47	
×2	27	
×1	20	
None	6	
Site of vector administration, %		
LAD	28	50
LAD/Cx	6	34
Cx	34	
Cx/right	14	
Right	6	
Ramus	6	16
RCA	6	
Time for vector administration, min	3.9±2.4 (2–10)	6.0±1.0 (4–8)
Postop hospitalization,‡ d	12.1±11.5 (5–40)	5.0±3.0 (3–11)

MI indicates myocardial infarction; CHE, history of congestive heart failure; Cx, circumflex; and RCA, right coronary artery.

Error estimates are mean±SD; range in parentheses, where applicable; % indicates percentage of population with relevant history.

*From cardiac catheterization assessment; if catheterization data not available, ejection fraction based on echocardiogram data.

†×1–3 indicates number of bypass grafts performed at time of surgery before vector administration.

‡For group A, for the 13 patients other than A5 and A15, average postoperative hospitalization was 10.4±9.2 days (range 5 to 36 days).

rus (Ad) gene-transfer vectors are advantageous for delivery of angiogens like VEGF in that Ad vectors provide a high transfection efficiency, remain highly localized, and express VEGF for a period of ≈1 to 2 weeks, which is sufficient to induce collateral vessels to relieve the ischemia but not long enough to evoke abnormal angiogenesis.[8–13]

Based on experimental animal models demonstrating the development of new blood vessels after in vivo administration of an Ad vector expressing human VEGF121 cDNA, including anatomic and functional correction of ischemia in a pig model of coronary obstruction,[8] the present study was directed toward evaluation of the administration of an $E1^-E3^-$ Ad vector (Ad_{GV}VEGF121.10) expressing the 121-amino-acid form of human VEGF to individuals with clinically significant coronary artery disease. The Ad_{GV}VEGF121.10 vector was administered directly to an ischemic area of the myocardium as an adjunct to conventional CABG surgery in a region that could not be bypassed (group A) or through a minithoracotomy as sole therapy (group B).

Methods

Ad_{GV}VEGF121.10

The Ad gene-transfer vector Ad_{GV}VEGF121.10 (GenVec, Inc) is based on the genome of the Ad5 serotype, with deletions in the E1 and E3 regions.[8] The expression cassette is in the E1 region and contains (right to left) the cytomegalovirus early/immediate enhancer/promoter, an artifical splice sequence, human VEGF121 cDNA, and the SV40 polyA/stop signal. Ad_{GV}VEGF121.10 was propagated in 293 cells, purified by CsCl density gradients, dialyzed, and stored at −70°C.[8,14] The vector met all safety criteria established by the Food and Drug Administration Bureau of Biologics (FDA BB) for clinical grade Ad vector preparations (FDA BB-IND 7381), including no detectable endotoxin or infectious agents and ≤1 replication-competent Ad for the total dose to be delivered.[15] The vector was titered in plaque-forming units (pfu)[14] and characterized as to particle units (pu) with the absorbance at 260 nm and the extinction coefficient for Ad (9.09×10^{-12} mL · particles^{-1} · cm^{-1}).[16] Just before use, the vector was thawed, diluted in a 3% sucrose solution, drawn up as 100 μL in 1-mL-insulin syringes with a 27-gauge needle (Becton Dickinson), and transported to the operating room.

Study Design

The study, which was approved by the local Institutional Review Board and the NIH DNA Recombinant Advisory Committee, was similar for groups A and B and included men and women aged 18 to 85 years with demonstrable reversible left ventricular ischemia as assessed by dobutamine stress echocardiography, rest and stress ^{99m}Tc-sestamibi nuclear medicine studies, and exercise tolerance testing. Twenty-four-hour Holter monitoring was used to exclude individuals with life-threatening arrhythmias. Other organ-specific inclusion criteria included room air Po_2 >60 mm Hg, Pco_2 <50 mm Hg, FEV_1 >1.2 L, hematocrit >30%, white blood cell count <10 000, serum urea nitrogen <40 U/L, and creatinine <2.5 g/dL. Group A (adjunct) had a requirement for an ejection fraction of ≥25% and ≥1 bypassable vessel, with the vector to be administered in a viable, ischemic region not amenable to bypass grafting. Group B (sole therapy) had a requirement of an ejection fraction ≥30% and included patients in whom CABG could not be performed due to lack of suitable bypass graft targets.

Ad_{GV}VEGF121.10 was administered by direct myocardial injection to both group A and B patients in a myocardial territory, irrespective of size, that demonstrated reversible ischemia by ^{99m}Tc-sestamibi perfusion scan with or without adenosine stress. The injections (100 μL/injection; 10 sites/patient; each site 1 to 1.5 cm apart) were administered to a region that extended from normal (bypassed) myocardium into the ischemic (nonbypassed) territory for collateral vessels that would bridge the myocardial territory from a patent inflow vessel to an ischemic territory and in which no continuous patent epicardial vessel was observed by angiography. For group A, once the CABG procedure was completed through a standard median sternotomy, the patient was rewarmed to 36°C, and the vector was administered to the myocardium while the patient was supported by partial bypass. Five dose groups were evaluated (n=3 patients per dose group), with total doses as follows: 4×10^8, $4\times10^{8.5}$, 4×10^9, $4\times10^{9.5}$, and 4×10^{10} pu. For group B (sole-therapy group, n=6), a small (4 to 5 cm) thoracotomy was used to expose the region of the myocardium chosen for vector administration. The vector (total dose 4×10^9 pu/patient) was then injected by direct visualization in the beating heart into a region of reversible ischemia.

General Safety Parameters

Blood parameters, including aspartate aminotransferase, alanine aminotransferase, bilirubin (total, direct, and indirect), alkaline phosphatase, albumin, white blood count, hemoglobin, hematocrit, platelet count, electrolytes, creatinine, serum urea nitrogen, lactate dehydrogenase, and creatine kinase (CK; CK-MB if the total CK was abnormal), were measured through the perioperative period and at days 14, 21, and 30 postoperatively.

Anti-Ad5 neutralizing antibody titers were assayed as previously described.[17] Plasma levels of VEGF were determined by standard ELISA; the assay detects all forms of human VEGF.[8,11] The samples were obtained in citrate tubes (Vacutainer L10278-00 2.7 mL; Becton Dickinson) to avoid contamination with platelet-derived VEGF.[18] Nose, throat, urine, and blood samples (before therapy and on days 2, 4, and 7) were evaluated for both $E1^-$ Ad vector and wild-type Ad.[15]

Cardiac-Specific Parameters

The degree of angina (on a scale of 1 to 4) was assessed preoperatively and 30 days after surgery by use of a questionnaire describing the Canadian Cardiovascular Society classification.[19] Serial ECG was used to assess myocardial ischemia, infarction, or arrhythmia. In the adjunct group, 24-hour Holter monitoring was performed before therapy and at 7 days after therapy.[20]

Biplanar contrast angiography was performed preoperatively within 2 months of the surgical procedure and at day 30 after therapy. The angiograms were reviewed by 3 interventional cardiologists blinded to treatment group and evaluated in the area of vector administration on the basis of Rentrop score (0 indicates no filling of collateral; 1, partial filling of branches of epicardial vessel; 2, partial filling of epicardial vessel; and 3, complete filling of epicardial vessel)[21] and collateral score (number of distinct collateral vessels contributing to the filling of an epicardial vessel in the region of vector administration).[8] All studies were read in random sequence, and samples from before and after the study were randomly presented to observers.

A 2-day combined rest-stress ^{99m}Tc-sestamibi study to assess myocardial viability was performed preoperatively within 2 weeks of the surgery and at 1 month after surgery. One hour after intravenous administration of ^{99m}Tc-sestamibi (25 to 30 mCi), ECG-gated single-photon emission computerized tomography (SPECT) imaging was performed with or without pharmacological stress with adenosine ($140\ \mu g \cdot kg^{-1} \cdot min^{-1}$ IV over 6 minutes). Semiquantitative analyses of perfusion were assessed by use of a 20-segment analysis (18 short axis and 2 long axis) in a blinded fashion by 2 nuclear cardiologists and scored in the region of vector administration on a scale of 0 to 4+, where 0 indicates no perfusion, 1 is severe hypoperfusion, 2 is moderate hypoperfusion, 3 is mild hypoperfusion, and 4 is normal perfusion. Using CEqual software (ADAC), we generated "bull's-eye" images for rest scans, stress scans, and their differences ("reversibility" of stress-induced ischemia) quantified as a percentage of the entire myocardium compared with a sex-matched normal database.[22]

Serial 2D echocardiography was used to determine the presence of pericardial effusion at baseline (within 2 weeks of operative procedure) and on days 2, 4, 7, 14, 21, and 30 postoperatively by a 0 to 3+ scale (0 indicates no effusion, 1 is mild effusion, 2 is moderate effusion, and 3 is large effusion). Regional wall motion at rest was assessed in the region of vector administration at baseline and on day 30 by an observer blinded to treatment groups, using a scale from 0 to 4+, where 0 indicates dyskinesis/akinesis, 1 is severe hypokinesis, 2 is moderate hypokinesis, 3 is mild hypokinesis, and 4 is normal.

Exercise tolerance testing was performed preoperatively and at day 30 according to a modified Bruce protocol.[23] Peak heart rate, peak heart rate×peak systolic blood pressure, and ST-segment/heart rate (ST/HR) slope (from peak exercise regression of ST depression expressed as a positive value referenced to heart rate) were determined.[23]

Statistical Analyses

Given that this is a phase I clinical trial, the number of patients at each dose in group A (n=3) and the total number of patients in group B (n=6) are too few to provide sufficient statistical power to discriminate within the variability of the various parameters that were assessed. Therefore, lack of statistical significance may not necessarily be interpreted as "no difference." The results are presented without formal error estimates and in the context of trends suggested by the data.

Results

Patient Demographics

The Ad_{GV}VEGF121.10 vector was administered to the myocardium as an adjunct to CABG (group A) in 15 patients (Table 1). The patients who were undergoing sole gene therapy (group B) were of similar age and had similar risk factors to those in group A but had a trend to a greater degree of cardiac disease, including an average lower ejection fraction and a higher proportion having undergone a prior CABG or angioplasty procedure.

Vector Administration

In group A, the region of injection was in the distribution of the left anterior descending (LAD) or circumflex coronary artery in the majority of patients, with the remainder in other sites (Table 1). One individual in group A (A10, vector dose $4\times10^{9.5}$ pu) underwent cardiopulmonary bypass, but no bypass graft was placed because of the severity of distal disease as assessed intraoperatively. In group B, 5 of 6 individuals were injected in either the LAD or circumflex territories. The average time required for injection was 3.9 minutes in group A and 6.0 minutes in group B; the time was longer in group B because of technical constraints imposed by the minimally invasive approach. Minimal extravasation of injectant was noted in both groups. Occasional premature ventricular beats were observed with insertion of the needle into the myocardium but were self-limited in all cases.

General Outcome

In group A, there were 2 perioperative (within 40 days of operative procedure) deaths. One, on postoperative day 40 in a 61-year-old male (A5, vector dose $4\times10^{8.5}$ pu) who was undergoing a third CABG operation, was related to a large anterior wall myocardial infarction secondary to occlusion of a graft to the LAD artery. Autopsy revealed a bacterial pneumonia and lung abscess; there were no abnormalities in the myocardial territory (posterior descending coronary artery) treated with the vector. The second death occurred on postoperative day 5 in an 85-year-old female (A15, vector dose 4×10^{10} pu) secondary to complications associated with an atheroembolic event in the ileocolic artery distribution. There was 1 additional sudden death of unknown cause (patient A14, dose 4×10^{10} pu, day 145 after therapy) in group A (mean±SD follow-up 286±76 days; range 175 to 414 days).

In group B, patients were extubated in the operating room, observed in the recovery room until awake, and transferred to the routine care floor until discharge. There were no perioperative or late deaths (mean±SD follow-up 170±17 days; range 149 to 196 days).

TABLE 2. CK Values of Patients Receiving Ad_{GV} VEGF121.10 (10^9 pu) as Sole Therapy

			Postoperative Day					
	Baseline		3		7		30	
Patients	CK, U/L	CK-MB	CK, U/L	CK-MB	CK, U/L	CK-MB	CK, U/L	CK-MB
Group A	85±47	5.7±0.0	479±547*	6.1±4.9†	107±71*	2.3±0.4†	65±46*	4.2±0.0†
Group B	153±115	...‡	339±305	...‡	190±91	...‡	103±52	...‡

*Patients A-5 and A-10 were excluded from analysis; A-5 had a large perioperative myocardial infarction, and A-10 had a compartment syndrome of the right lower leg.

†Patient A-5 was excluded from analysis because of a large perioperative myocardial infarction.

‡CK-MB was not assessed if total CK was within normal range.

General Safety Parameters

There was no evidence of a dose-related trend toward abnormalities in any blood parameters in group A and no differences in any blood parameters for group B at day 3, 7, or 30 compared with before therapy. Plasma VEGF levels were evaluated over a 30-day period after therapy in the individuals who received $4\times10^{9.5}$ and 4×10^{10} pu in the adjunct group and in all patients in the sole-therapy group. There were no trends to increases above baseline levels except at day 3, when the average value was 158 pg/mL. There was no evidence of acute or sustained hypotension or hemodynamic compromise associated with sole therapy (group B). In both groups, serum anti-Ad5 neutralizing antibody levels were increased in all individuals, although more so in patients with higher pretherapy anti-Ad5 neutralizing antibodies (not shown). No shedding of vector or wild-type Ad was detected in any sample from any site in any patient (group A total 234 samples; group B total 71 samples).

Cardiac-Related Parameters

In group A, there was no dose-related trend of an increase in CK related to vector administration (Table 2). In group B, there was no increase in CK after therapy (Table 2). In either group, daily ECG during hospitalization and at 14 and 30 days showed no new ST changes or Q waves (Table 3). In group A, 24-hour Holter monitoring performed before therapy and at day 7 demonstrated no average increase in supraventricular or ventricular arrhythmias after therapy. Serial echocardiographic studies in both groups demonstrated no evidence of significant (≥2) pericardial effusions. In both groups, resting echocardiographic assessment at day 30 compared with before therapy showed no regional wall motion abnormalities in the territory where the vector was administered.

Assessment of angina class in group A showed improvement in all individuals evaluated, but this cannot be attributed specifically to the Ad_{GV}VEGF121.10 therapy because of the effects of bypass. However, in all 6 of the individuals in group B, there was a decrease in angina classification at day 30 compared with before therapy (Figure 1).

Coronary angiograms obtained 30 days after vector administration demonstrated no hemangiomas or other pathological vascular structures. In group A, a majority of the blinded observations demonstrated an improvement in Rentrop scores in the area of vector administration after therapy compared with before therapy (Figure 2A). The collateral scores in group A demonstrated a similar trend (Figure 2B). Likewise, in group B, in which no CABG was performed that might provide a watershed effect in the area treated with the vector, a majority of the Rentrop and collateral score observations

TABLE 3. Comparison of ECG at Baseline and Postvector Day 30 for Patients Receiving Ad_{GV} VEGF121.10

Group and Patient	Vector Dose, pu	Atrial Fibrillation	New MI	New Ischemia	Any New Abnormality
A					
A1, A2, A4, A6–A14	4×10^8–4×10^{10}	0	0	0	0
A3	4×10^8	0	Yes*	0	0
A5	$4\times10^{8.5}$	0	Yes†	Yes†	Yes†
A15	4×10^{10}	0	0	0	Yes‡
B					
B1, B4–B6	4×10^9	0	0	0	0
B2	4×10^9	0	0§	0§	0§
B3	4×10^9	0	0	Yes‖	0

MI indicates myocardial infarction.

*Possible anteroseptal MI, although this change could be attributed to change in lead placement.

†Anterolateral MI.

‡Loss of previously seen repolarization abnormality.

§Loss of preoperative anterior MI pattern.

‖Anterolateral ischemia.

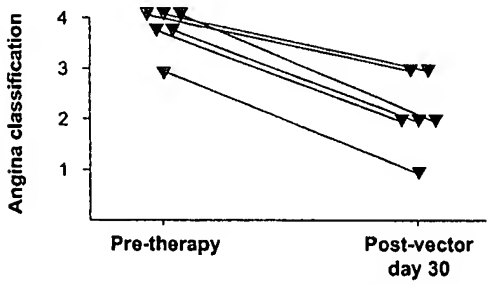


Figure 1. Canadian angina classifications before and 30 days after intramyocardial delivery of $Ad_{GV}VEGF121.10$ (4×10^9 pu) as sole therapy. Each before-and-after pair represents 1 individual. Only a single dose was used.

showed a similar trend of improvement after therapy compared with before therapy (Figure 2C).

For group A assessed as a single cohort, semiquantitative analysis of the ^{99m}Tc-sestamibi images in the area of vector administration showed no changes in relative blood flow at 30 days after vector administration compared with pretherapy at rest or after adenosine-induced stress. Likewise, for group B evaluated as a cohort, analysis of the sestamibi images demonstrated no differences in relative blood flow in the area of vector administration at rest or after adenosine-induced stress. Interestingly, analysis of the sestamibi images for wall motion at stress in the region of vector administration showed an improvement at 30 days after vector administration in the majority of patients, both in group A (66% [8/12] improved) and in group B (66% [4/6] improved). For group B, in which bull's-eye analyses of the sestamibi scans could be performed with assessment of vector administration as the only variable, 4 of 6 individuals showed an improvement 30 days after therapy in the proportion of myocardium that showed reversibility (reversible stress-induced ischemia) before therapy (70±31%) versus 30 days after therapy (54±36%).

Assessment of treadmill exercise in group A showed no differences (30 days after vector compared with before therapy) in exercise duration, heart rate×blood pressure, or ST/HR slope. For group B, in which vector administration was the only therapy, assessment of treadmill exercise showed an improvement in exercise duration in 50% (3/6), in peak heart rate×blood pressure in 50% (3/6), and in ST/HR slope (ie, lower values) in 75% (3/4; data not available in 2 secondary to right bundle-branch block precluding analysis; Figure 3).

Discussion

The development of strategies to deliver angiogens to revascularize the ischemic myocardium without the need for mechanical manipulation of atherosclerotic vessels is potentially of profound importance in the treatment of coronary artery disease. The present study demonstrates that it is feasible to safely use an adenovirus gene-transfer vector to deliver the coding sequence of the 121-amino-acid form of the human VEGF angiogen to the myocardium of individuals with clinically significant coronary artery disease. Based on the knowledge that VEGF plays a critical role in embryonic cardiac angiogenesis[24] and on preclinical studies that demonstrate that the $Ad_{GV}VEGF121.10$ gene-transfer vector will induce functional angiogenesis in the ischemic myocardium of experimental animals,[8] the results of the present study provide encouragement that this strategy may be useful in revascularizing the ischemic heart in humans.

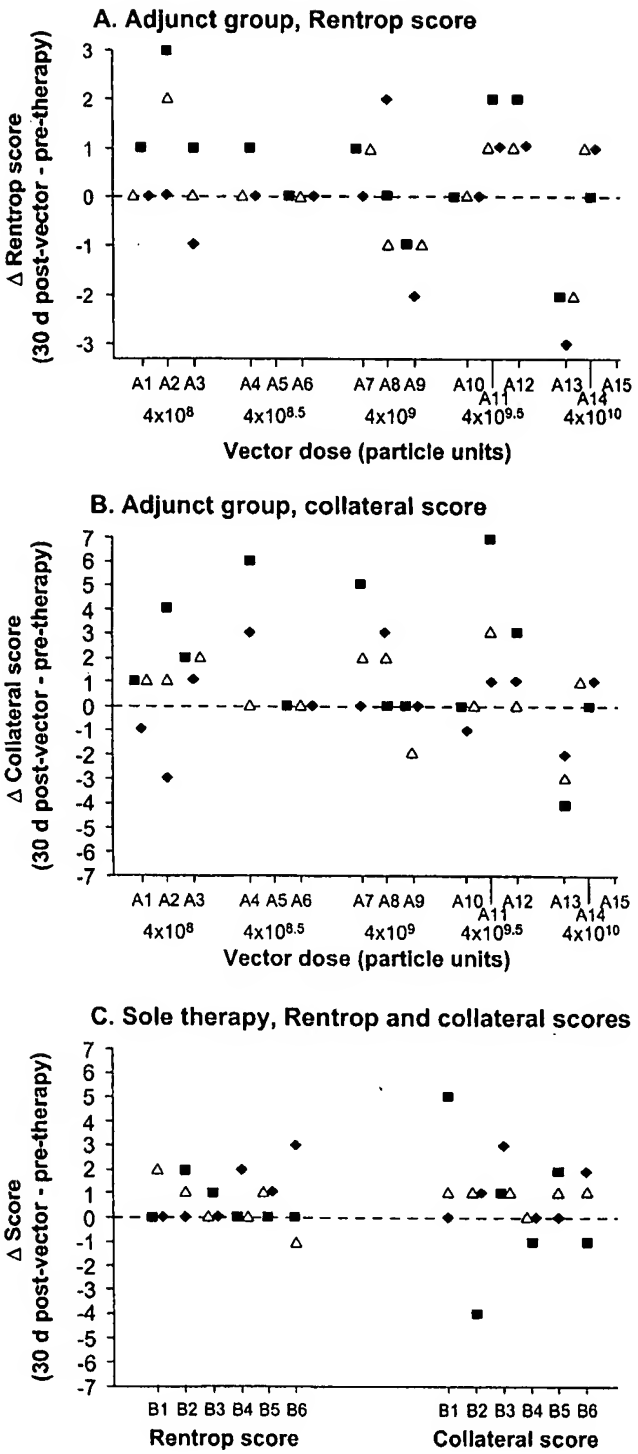


Figure 2. Semiquantitative, blinded assessment of coronary angiograms before and 30 days after intramyocardial administration of $Ad_{GV}VEGF121.10$. A, Rentrop scores, adjunct to CABG. B, Collateral scores, adjunct to CABG. C, Rentrop and collateral scores, sole therapy/minithoracotomy. For all panels, a positive value (score at 30 days after therapy minus before therapy) indicates improvement at 30 days compared with before therapy. ■, Observer 1; △, observer 2; ◆, observer 3.

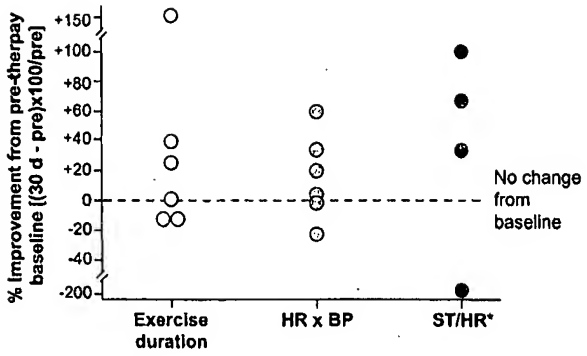


Figure 3. Treadmill exercise before and 30 days after intramyocardial administration of Ad_{GV}VEGF121.10 in group B (sole therapy). Shown is percent improvement for each patient at 30 days after vector compared with baseline [(30-days posttherapy−pretherapy)×100/pretherapy] for duration of exercise, blood pressure×heart rate (HR x BP), and ST-segment depression/heart rate (ST/HR). *ST/HR slope plotted as negative of percent change from baseline; ie, a positive score reflects a reduction in test value to a less abnormal result.

Cardiac-Specific Parameters

There was no evidence of either myocardial inflammation or necrosis, as was shown by the lack of dose-related increases in CK, arrhythmias or ST/T wave changes assessed by Holter and ECG monitoring, and deterioration of global or segmental function in the area of vector administration as assessed by echocardiography or ^{99m}Tc-sestamibi. There was no evidence of excess deranged angiogenesis, as evidenced by no hemangiomas or otherwise deranged vasculature in the coronary angiograms and no evidence of myocardial edema or pericranial effusions. These observations are consistent with the assessment of the safety of administration of human VEGF165 cDNA plasmids to the human myocardium, either by intracoronary administration[25] or by direct myocardial administration.[9]

The present study is limited by the number of cases being too small to provide sufficient power to discriminate within the variability of the various methods used to assess cardiac function. Furthermore, although the vector was delivered in group A to a myocardial territory that could not be bypassed, the vector was administered in conjunction with a conventional CABG procedure, and thus it is impossible to exclude the possibility of CABG-related watershed perfusion affecting the region of vector administration. Despite these constraints, the trends of several of the efficacy-related parameters assessed 30 days after therapy are encouraging. First, all patients had improvement in their angina classification. Although this can be ascribed to the CABG procedure in group A, there was a similar trend to improvement in the sole-therapy group. Second, in the majority of individuals in both groups A and B, angiographic studies showed increased coronary artery filling and/or number of collaterals in the region of vector administration. Third, the majority of individuals in groups A and B had improvement in ventricular wall motion with stress as assessed by ^{99m}Tc-sestamibi scans. Finally, the majority of individuals in the sole-therapy group had evidence of decreased stress-induced reversible ischemia on sestamibi perfusion scans, as well as improvements in treadmill exercise parameters. The observed decrease in reversible ischemia could theoretically be caused by infarction of this territory, but this is unlikely, because there were no corresponding infarction-related changes in CK, ECG or echocardiography.

Systemic Parameters

Assessment of blood and urine parameters suggested no systemic abnormalities related to the vector, consistent with the general clinical experience with E1$^-$ Ad gene transfer to humans.[26,27] Importantly, there was no evidence of liver function abnormalities as a function of vector dose; this is important because the liver is a major site of Ad vector–induced inflammation at high doses in some studies in experimental animals.[28,29] One explanation for this lack of systemic toxicity in the human studies is that the vector preparations used in clinical studies are highly purified (<1 replication-competent Ad [RCA] per total dose), in contrast to laboratory-grade vectors, which are often contaminated with RCAs.[15]

Myocardial administration of Ad_{GV}VEGF121.10 induced an increase in anti-Ad neutralizing antibodies in most of the study population. This was more pronounced in individuals with detectable serum anti-Ad neutralizing antibodies before therapy, consistent with the vector inducing a memory immune response against subgroup C Ad.[15,29,30] Despite this, there was no evidence of systemic immune-related toxicity in any patient, including no immediate anaphylactic-type reactions, vasculitis, or renal damage.

Finally, it is known that systemic administration of the VEGF protein at high doses results in systemic hypotension in experimental animals and humans.[31] However, the present study demonstrated no large increases in VEGF levels in the systemic circulation after myocardial administration of Ad_{GV}VEGF121.10 and no hypotension attributable to the vector, consistent with experimental animal studies using Ad_{GV}VEGF121.10.[8]

Future Role of Angiogenic Gene Therapy

The ability to biologically revascularize tissues, if proven to be safe and efficacious in large, controlled trials, will be an invaluable treatment for patients with diffuse disease not amenable to conventional CABG or PTCA and may be useful as initial therapy in some individuals in place of routine CABG or PTCA therapy. In the present study, we used an Ad vector to deliver the VEGF121 cDNA. As an alternative, Losordo et al[9] used myocardial administration of a VEGF165 plasmid as sole therapy for myocardial ischemia and demonstrated a safety profile and trends in efficacy parameters similar to our study. If one assumes that the neovasculature induced by angiogenic therapy is persistent and physiologically relevant, the small-caliber vessels generated by this therapy may furthermore be relatively spared from the effects of atherosclerosis, which primarily affects larger vessels. Finally, given the decreased survival overall and decreased angina-free survival noted in patients in whom incomplete revascularization is accomplished, the advantages of providing "complete" revascularization in patients undergoing standard CABG or PTCA may also prove be a significant benefit of this new therapy.

Acknowledgments

These studies were supported in part by NIH R01 HL-57318 and M01RR00047; Jeffry M. and Barbara Picower Foundation, Palm Beach, Fla; Will Rogers Memorial Fund, Los Angeles, Calif; GenVec, Inc, Rockville, Md; and Parke-Davis Pharmaceutical Research, Warner-Lambert Company, Ann Arbor, Mich. We would like to thank Stephanie Rempel and Sharon Bauer for help with the study and N Mohamed for help in preparation of this manuscript.

References

1. Rosengart TK, Patel SR, Crystal RG. Therapeutic angiogenesis: protein and gene therapy delivery strategies. *J Cardiovasc Res.* 1999;6:29–40.
2. Simons M, Ware JA. Food for starving hearts. *Nat Med.* 1996;2:519–520.
3. Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E. Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. *Cardiovasc Res.* 1994;28:1176–1179.
4. Li J, Brown LF, Hibberd MG, Grossman JD, Morgan JP, Simons M. VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. *Am J Physiol.* 1996;270:H1803–H1811.
5. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. *J Cell Biochem.* 1991; 47:211–218.
6. Folkman J, Haudenschild C. Angiogenesis in vitro. *Nature.* 1980;288: 551–556.
7. Thomas KA. Vascular endothelial growth factor, a potent and selective angiogenic agent. *J Biol Chem.* 1996;271:603–606.
8. Mack CA, Patel SR, Schwarz EA, Zanzonico P, Hahn RT, Ilercil A, Devereux RB, Goldsmith SJ, Christian TF, Sanborn TA, Kovesdi I, Hackett N, Isom OW, Crystal RG, Rosengart TK. Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. *J Thorac Cardiovasc Surg.* 1998;115:168–176; discussion 176–177.
9. Losordo DW, Vale PR, Symes JF, Dunnington CH, Esakof DD, Maysky M, Ashare AB, Lathi K, Isner JM. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. *Circulation.* 1998; 98:2800–2804.
10. Magovern CJ, Mack CA, Zhang J, Hahn RT, Ko W, Isom OW, Crystal RG, Rosengart TK. Direct in vivo gene transfer to canine myocardium using a replication-deficient adenovirus vector. *Ann Thorac Surg.* 1996; 62:425–433; discussion 433–434.
11. Mack CA, Magovern CJ, Budenbender KT, Patel SR, Schwarz EA, Zanzonico P, Ferris B, Sanborn T, Isom P, Isom OW, Crystal RG, Rosengart TK. Salvage angiogenesis induced by adenovirus-mediated gene transfer of vascular endothelial growth factor protects against ischemic vascular occlusion. *J Vasc Surg.* 1998;27:699–709.
12. Muhlhauser J, Merrill MJ, Pili R, Maeda H, Bacic M, Bewig B, Passaniti A, Edwards NA, Crystal RG, Capogrossi MC. VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis in vivo. *Circ Res.* 1995;77:1077–1086.
13. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. *Mol Cell.* 1998;2:549–558.
14. Rosenfeld MA, Yoshimura K, Trapnell BC, Yoneyama K, Rosenthal ER, Dalemans W, Fukayama M, Bargon J, Stier LE, Stratford-Perricaudet L, Perricaudet M, Guggino WB, Pavirani A, Lecocq J-P, Crystal RG. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 1992;68:143–155.
15. Crystal RG, McElvaney NG, Rosenfeld MA, Chu CS, Mastrangeli A, Hay JG, Brody SL, Jaffe HA, Eissa NT, Danel C. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat Genet.* 1994;8:42–51.
16. Mittereder N, March KL, Trapnell BC. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J Virol.* 1996;70: 7498–7509.
17. Mack CA, Song WR, Carpenter H, Wickham TJ, Kovesdi I, Harvey BG, Magovern CJ, Isom OW, Rosengart T, Falck-Pedersen E, Hackett NR, Crystal RG, Mastrangeli A. Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. *Hum Gene Ther.* 1997;8:99–109.
18. Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C, Selby PJ. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. *Br J Cancer.* 1998;77:956–964.
19. Campeau L. Grading of angina pectoris. *Circulation.* 1976;54:522–523. Letter.
20. Kligfield P, Hochreiter C, Kramer H, Devereux RB, Niles N, Kramer-Fox R, Borer JS. Complex arrhythmias in mitral regurgitation with and without mitral valve prolapse: contrast to arrhythmias in mitral valve prolapse without mitral regurgitation. *Am J Cardiol.* 1985;55:1545–1549.
21. Rentrop KP, Cohen M, Blanke H, Phillips RA. Changes in collateral channel filling immediately after controlled coronary artery occlusion by an angioplasty balloon in human subjects. *J Am Coll Cardiol.* 1985;5: 587–592.
22. Williams KA, Taillon LA. Reversible ischemia in severe stress technetium 99m-labeled sestamibi perfusion defects assessed from gated single-photon emission computed tomographic polar map Fourier analysis. *J Nucl Cardiol.* 1995;2:199–206.
23. Okin PM, Kligfield P. Heart rate adjustment of ST segment depression and performance of the exercise electrocardiogram: a critical evaluation. *J Am Coll Cardiol.* 1995;25:1726–1735.
24. Hanahan D. Signaling vascular morphogenesis and maintenance. *Science.* 1997;277:48–50.
25. Laitinen M, Hartikainen J, Eranen J, Kiviniemi M, Hiltunen MO, Laakso M, Ya-Herttuala S. Catheter-mediated VEGF gene transfer to human coronary arteries after angioplasty: safety results from phase I Kuopio angioplasty gene transfer trial (KAT trial). *Circulation.* 1998;98(suppl I):I-322. Abstract.
26. Harvey B-G, Hackett N-R, El-Saw YT, Rosengart TK, Hirschowitz EA, Lieberman MO, Lesser ML, Crystal RG. Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. *J Virol.* In press.
27. Ohwada A, Hirschowitz EA, Crystal RG. Regional delivery of an adenovirus vector containing the *Escherichia coli* cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum Gene Ther.* 1996;7:1567–1576.
28. Gao GP, Yang Y, Wilson JM. Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J Virol.* 1996;70:8934–8943.
29. Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum Gene Ther.* 1997;8:37–44.
30. Wilson JM. Adenoviruses as gene-delivery vehicles. *N Engl J Med.* 1996;334:1185–1187.
31. Hariawala MD, Horowitz JJ, Esakof D, Sheriff DD, Walter DH, Keyt B, Isner JM, Symes JF. VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts. *J Surg Res.* 1996;63:77–82.

ANNALS OF SURGERY
Vol. 230, No. 4, 466–472

Six-Month Assessment of a Phase I Trial of Angiogenic Gene Therapy for the Treatment of Coronary Artery Disease Using Direct Intramyocardial Administration of an Adenovirus Vector Expressing the VEGF121 cDNA

Todd K. Rosengart, MD,* Leonard Y. Lee, MD,*† Shailen R. Patel, MD,*† Paul D. Kligfield, MD,‡ Peter M. Okin, MD,‡ Neil R. Hackett, PhD,† O. Wayne Isom, MD,* and Ronald G. Crystal, MD†

*From the *Department of Cardiothoracic Surgery, †Division of Pulmonary and Critical Care Medicine, and ‡Division of Cardiology, Weill Medical College of Cornell University—New York Presbyterian Hospital, New York City, New York*

Objective

To summarize the 6-month follow-up of a cohort of patients with clinically significant coronary artery disease who received direct myocardial injection of an $E1^-E3^-$ adenovirus (Ad) gene transfer vector (Ad_{GV}VEGF121.10) expressing the human vascular endothelial growth factor (VEGF) 121 cDNA to induce therapeutic angiogenesis.

Background

Therapeutic angiogenesis describes a novel approach to the treatment of vascular occlusive disease that uses the administration of growth factors known to induce neovascularization of ischemic tissues.

Methods

Direct myocardial injection of Ad_{GV}VEGF121.10 into an area of reversible ischemia was carried out in 21 patients as an adjunct to conventional coronary artery bypass grafting (group A, n = 15) or as sole therapy using a minithoracotomy (group B, n = 6).

Results

No evidence of systemic or cardiac-related adverse events related to vector administration was observed up to 6 months after therapy. Trends toward improvement in angina class and exercise treadmill testing at 6-month follow-up in the sole therapy group suggest the effects of this therapy are persistent for ≥6 months.

Conclusions

This study suggests that direct myocardial administration of Ad_{GV}VEGF121.10 appears to be well tolerated in patients with clinically significant coronary artery disease. Initiation of phase II evaluation of this therapy appears warranted.

Therapeutic angiogenesis is a novel experimental strategy for treating myocardial ischemia in which neovascularization of ischemic tissues is accomplished by the administration of mediators known as "angiogens" that induce the formation of blood vessels.[1,2] This strategy is based on the following observations:

1. The native biologic response to vascular occlusion involves the formation of collateral vessels that serve to bypass these obstructions.
2. Upregulation of the expression of naturally occurring angiogens and their cognate receptors appears to underlie the process of collateral vessel formation.
3. The native process of collateral vessel formation is characteristically incomplete in relieving ischemia

Presented at the 119th Annual Meeting of the American Surgical Association, April 15–17, 1999, Hyatt Regency Hotel, San Diego, California.
Supported in part by NIH grants R01 HL 57318 and M01RR00047; Jeffry M. and Barbara Picower Foundation, Palm Beach, Florida; Will Rogers Memorial Fund, Los Angeles, California; GenVec, Inc., Rockville, Maryland; and Parke-Davis Pharmaceutical Research, Warner-Lambert Company, Ann Arbor, Michigan.
Correspondence: Todd K. Rosengart, MD, Dept. of Cardiothoracic Surgery, Weill Medical College of Cornell University—New York Presbyterian Hospital, New York, NY 10021.
Accepted for publication April 1999.

secondary to atherosclerotic vascular occlusive disease.[3,4]

Percutaneous transluminal coronary angioplasty and coronary artery bypass grafting (CABG), the current interventional therapies for treating atherosclerotic coronary artery disease (CAD), are of limited benefit in patients with severe or diffuse disease and do not adequately address the issue of restenosis. Therapeutic angiogenesis may therefore be a valuable adjunct to conventional interventional strategies for treating CAD.

Of the many polypeptide angiogens implicated as catalysts of angiogenesis, vascular endothelial growth factor (VEGF), a protein coded by a seven-exon gene localized on chromosome 6, appears to be one of the most important. In this context, deletion of the VEGF and VEGF receptor genes in knockout models results in lethal embryonic abnormalities, and VEGF has been demonstrated to induce vigorous collateral vessel formation in several models.[1–8]

One approach to therapeutic angiogenesis is gene therapy, a drug-delivery strategy by which the coding sequences for specific angiogens can be delivered to targeted tissues such as the myocardium to enable cells making up the tissue to produce and secrete a selected angiogen such as VEGF.[7–9] Replication-deficient recombinant adenovirus (Ad) gene transfer vectors have been shown in animal models to be particularly advantageous for delivering angiogens such as VEGF to target tissues such as the myocardium, in that Ad vectors provide high levels of localized expression of the angiogen for approximately 1 to 2 weeks, a duration demonstrated in experimental animals to be sufficient to induce angiogenesis but not long enough to evoke abnormal blood vessel formation.[7,10–13]

The present study evaluates the administration of Ad_{GV}-VEGF121.10, an $E1^-E3^-$ Ad vector expressing the 121-amino-acid form of human VEGF, to patients with clinically significant CAD. We have previously reported the early (30-day) results with this cohort, in which this therapy appeared to be well tolerated.[14] This report summarizes the 6-month results of this phase I trial.

METHODS

Ad_{GV}VEGF121.10

The clinical-grade Ad gene transfer vector Ad_{GV}-VEGF121.10 (GenVec, Inc., Rockville, MD) is made up of an Ad5 serotype backbone, with deletions in the E1 and E3 regions.[7] The expression cassette in the E1 region contains (right to left orientation) the cytomegalovirus early/immediate enhancer/promoter, an artificial splice sequence, the human VEGF121 cDNA, and the SV40 polyA/stop signal. Ad_{GV}VEGF121.10 was produced and stored at −70°C as previously described.[14,15] At the time of vector delivery, the vector was thawed, immediately diluted, drawn up as 100 μl in 1-ml insulin syringes with a 27-gauge needle (Becton Dickinson, Franklin Lakes, NJ), and placed into a sterile container for transport to the operating room.

Study Design

Two groups were evaluated. In group A (adjunct group), the Ad_{GV}VEGF121.10 vector was administered during conventional CABG surgery by direct intramyocardial injection to an ischemic area that could not be bypassed. In the patients in group B (sole therapy), in whom CABG could not be carried out because there was a lack of suitable bypass graft targets, the vector was administered as a sole therapy by direct intramyocardial injection through a minithoracotomy to an area of ischemic myocardium.[14] The inclusion criteria for groups A and B included men and women ages 18 to 85 with demonstrable reversible left ventricular ischemia, as assessed by rest and stress ^{99m}Tc-sestamibi nuclear medicine studies. Twenty-four-hour Holter monitoring was used to exclude patients with life-threatening arrhythmias. Other inclusion criteria included room air $Po_2 > 60$ torr, $Pco_2 < 50$ torr, forced expiratory volume in 1 second > 1.2 L, hematocrit >30%, white blood cell count <10,000, blood urea nitrogen <40 U/L, and creatinine <2.5 g/dl. Exclusion criteria included ejection fraction <25% for group A and <30% for group B.

Ten injections of Ad_{GV}VEGF121.10 (100 μl per injection; each site 1 to 1.5 cm apart) were administered by direct myocardial injection to both group A and B patients in a single coronary myocardial territory demonstrating reversible ischemia by ^{99m}Tc-sestamibi perfusion scan with or without adenosine stress. For group A, the CABG procedure was performed through a standard median sternotomy, with the vector administered directly to the myocardium during cardiopulmonary bypass, but after rewarming to 36°C after the completion of bypass grafting. Group A total doses were increased in half-log increments from 4×10^8 particle units (pu) to 4×10^{10} pu (n = 3 per dose group). For group B, the myocardium was reached through a small (4- to 5-cm) thoracotomy. The vector (total dose 4×10^9 pu per patient) was injected under direct visualization into the beating heart into the region of reversible ischemia identified by ^{99m}Tc-sestamibi scanning.

General Safety Parameters

Routine blood parameters including complete blood count (white blood count, hemoglobin, hematocrit, and platelet count), electrolytes, creatinine, blood urea nitrogen, and lactic dehydrogenase were used as indirect measures of systemic toxicity. With the knowledge that 90% of Ad vectors delivered systemically in animal models are taken up by the liver, liver function tests were serially evaluated, including aspartate transferase, alkaline transferase, bilirubin (total, direct, indirect), alkaline phosphatase, and albumin. Systemic vector-specific parameters included anti-Ad5 neutralizing antibody titers by wild type Ad5, as previously described.[16,17]

Table 1. SERUM PARAMETERS COMPARING BASELINE TO 6 MONTHS AFTER SURGERY*

Parameter	Baseline	3-Month Follow-Up	6-Month Follow-Up
CPK (μg/l)	172 ± 117	160 ± 91	115 ± 34
BUN (mg/dl)	19 ± 5	19 ± 6	20 ± 5
Creatinine (mg/dl)	0.9 ± 0.3	0.9 ± 0.1	0.9 ± 0.3
Hgb (g/dl)	13 ± 2	13 ± 2	12 ± 2
Hct (%)	38 ± 4	38 ± 4	38 ± 5
WBC ($\times 10^3$ μl)	6.0 ± 1.0	6.0 ± 0.5	5.5 ± 1.1
ALT (SGPT) (U/L)	24 ± 9.0	—	19 ± 6
AST (SGOT) (U/L)	20 ± 2.0	—	17 ± 4
Alk Phos (U/L)	81 ± 23	81 ± 18	84 ± 21
Bilirubin (mg/dl)	0.8 ± 0.3	0.6 ± 0.2	0.6 ± 0.2

CPK, creatinine phosphokinase; BUN, blood urea nitrogen; Hgb, hemoglobin; Hct, hematocrit; WBC, white blood cell count; ALT, alkaline transferase (SGPT, serum glutamic pyruvic transferase); AST, aspartate transferase (SGOT, serum glutamic oxaloacetic transferase); Alk Phos, alkaline phosphatase.
* In patients receiving intramyocardial Ad_{GV} VEGF121.10 as sole therapy.

Cardiac-Specific Parameters

Several parameters were examined at 6 months to assess the cardiac-specific effects of direct myocardial injection of Ad_{GV}VEGF121.10 to compare the previously reported preoperative and 1 month posttherapy data.[14] Creatine phosphokinase (CPK; CPK-MB if the total level of CPK was abnormal) was used as a measure of cardiac toxicity. Evidence of myocardial ischemia, infarction, or arrhythmia was assessed by serial electrocardiography. The degree of angina was assessed by direct questioning of the patient, using a 1 to 4 scale according to the Canadian Cardiovascular Society classification.[18] Weekly nitroglycerine intake was similarly determined.

Exercise tolerance testing was performed according to a modified Bruce protocol.[19] Peak heart rate, peak heart rate × peak systolic blood pressure, and ST/HR slope (maximal rate of change of ST depression with respect to heart rate, by linear regression) were determined using conventional methodology.[19]

Statistical Analyses

The number of patients at each dose in group A (n = 3) and the total number of patients in group B (n = 6) are too small to provide sufficient statistical power to discriminate within the variability of the various parameters that were assessed. Thus, lack of statistical significance may not necessarily be interpreted as "no difference." In this context, the results are presented without formal error estimates and are presented in the context of trends suggested by the data.

RESULTS

General Outcome

The demographics and cardiac risk factors of these patients have been reported previously and were typical of the general CABG population.[14] All coronary territories were treated in group A, whereas injections in group B were limited to the left ventricle free wall. Vector administration was well tolerated in both patient groups.[14] There were no complications or late deaths related to vector administration in either cohort. There have been no additional deaths other than the three originally reported.[14] Patients A13 (adjunct group, dose 4×10^{10} pu) and B1 (sole therapy group, dose 4×10^{9} pu) underwent subsequent percutaneous transluminal coronary angioplasty, and patient B1 underwent subsequent CABG on postoperative day 130.

No abnormalities were found in any blood tests at the analysis performed 3 or 6 months after surgery (Table 1). Serum anti-Ad5 neutralizing antibody levels had decreased toward baseline by 6 months (Fig. 1).

Cardiac-Related Parameters

In both groups, there were no interval changes in the electrocardiogram after the perioperative period (Table 2), and there was no increase in CPK above baseline values at 3 or 6 months of follow-up (see Table 1). Due to the effect

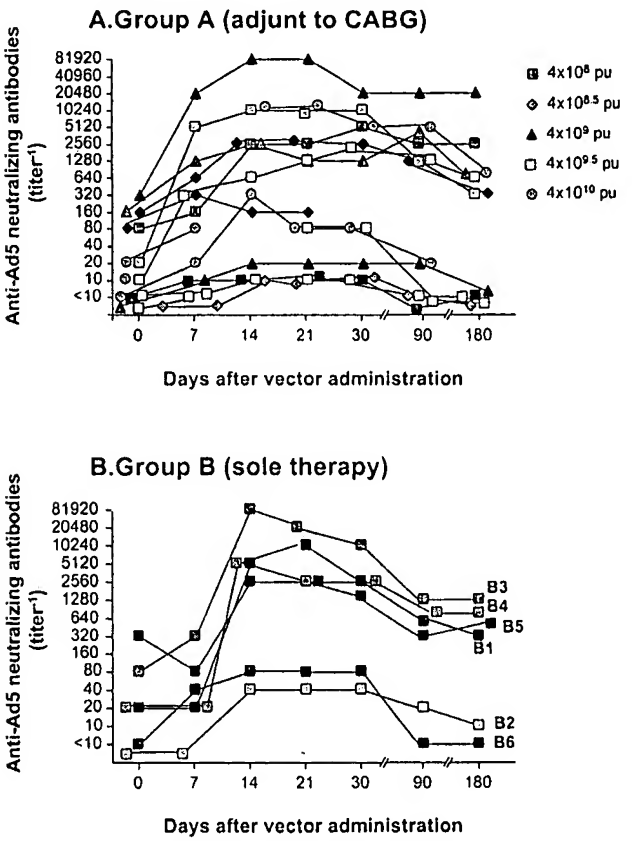


Figure 1. Assessment of anti-Ad5 neutralizing antibody titers^{-1} before therapy and after intramyocardial administration of Ad_{GV}VEGF121.10. (A) Patients receiving Ad_{GV}VEGF121.10 as an adjunct to CABG. (B) Patients receiving Ad_{GV}VEGF121.10 as sole therapy. Data are presented as titer^{-1}. Data for group A have been previously reported for the 4×10^{8} pu dose ≤90 days and for all other doses ≤30 days (Harvey et al 1999[17]).

Table 2. EVALUATION OF ELECTROCARDIOGRAM CHANGES COMPARING BASELINE TO 6 MONTHS AFTER SURGERY*

Patient	New ST-T Wave Changes	New Q Waves	New Infarcts	Other
B1	None	None	No	No
B2	None	None	No	No
B3	None	None	No	No
B4	None	None	No	Change in conduction†
B5	Inferolateral ischemia*	None	No	Bradycardia
B6	None	None	No	Change in repolarization†

* In patients receiving intramyocardial Ad_{GV} VEGF121.10 as sole therapy.
† Not in the region of vector administration.

of CABG, the relation of angina class in group A patients to the Ad_{GV}VEGF121.10 administration cannot be interpreted. However, in all six patients in group B in whom no additional interventional therapies had been performed, there was a decrease in angina classification at 1 and 6 months compared to before therapy (Fig. 2A). A corresponding persistent decrease in sublingual nitroglycerin use was noted in five of the six patients, in the setting of stable dosages of other antianginal medications (Fig. 2B).

Assessment of treadmill exercise in the patients in group B demonstrated a trend toward a decrease in ST/HR slope, suggesting decreased myocardial ischemia at 6 months compared with baseline (Table 3). The extent of exercise and BP × HR remained stable.

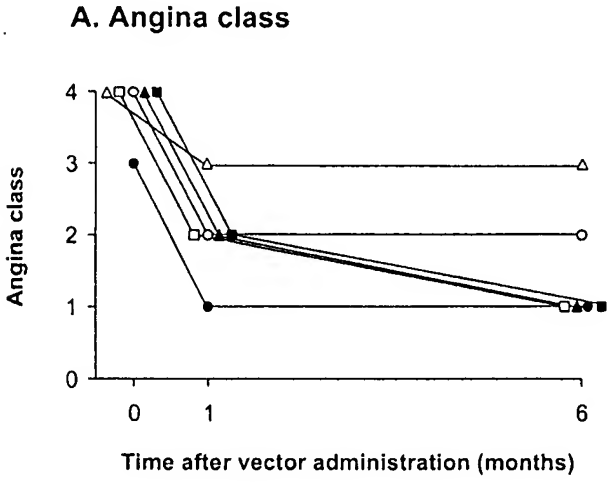
DISCUSSION

The present study demonstrates that the administration of an Ad vector delivering the coding sequence of the 121-amino-acid form of human VEGF to the myocardium of patients with clinically significant CAD is well tolerated during a 6-month period. Based on the known biologic activity of VEGF and the limited persistence of transgene expression produced by this vector, it would appear to be reasonable to conclude, at least based on this small cohort, that no adverse effects can be anticipated with this therapy. Although the number of patients in this study is too small to ensure a similar outcome in larger studies or in general use, and these early results are too preliminary to substantiate efficacy, the trends in this study of the persistence of apparently beneficial effects without apparent toxicity 6 months after therapy are encouraging. We conclude that direct myocardial administration of Ad_{GV}VEGF121.10 appears to be well tolerated in patients with clinically significant CAD, and initiation of phase II evaluation of this therapy appears warranted.

Cardiac-Specific Parameters

On a theoretical basis, chronic inflammation in response to the Ad gene transfer vector represents the primary long-term risk to the heart after direct myocardial administration of Ad_{GV}VEGF121.10. However, there was no evidence of either chronic myocardial inflammation or necrosis after therapy, as evidenced by the absence of increases in CPK at 3 and 6 months, and arrhythmias or other abnormalities assessed by electrocardiography.

Because it is impossible to exclude the possibility of CABG-related "watershed" perfusion affecting the region of vector administration in group A patients, analysis of the persistence of cardiac-related efficacy after direct myocardial administration of the Ad_{GV}VEGF121.10 vector in the present study was limited to group B. Although the group B cohort is too small to analyze statistically, group B patients



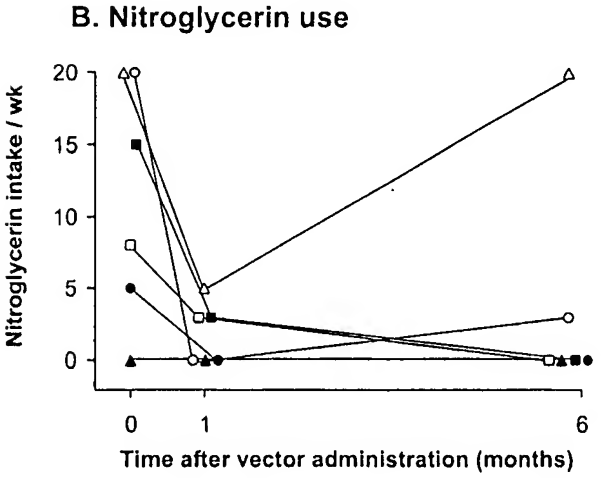


Figure 2. Assessment of angina before therapy and 1 and 6 months after intramyocardial administration of Ad_{GV}VEGF121.10 in the sole therapy/minithoracotomy group. (A) Canadian Cardiovascular Society angina classifications before and 1 and 6 months after Ad_{GV}-VEGF121.10 therapy. (B) Sublingual nitroglycerin use assessed on a weekly basis. Pretherapy and 30-day data have been previously reported (Rosengart et al 1999[14]).

Table 3. EVALUATION OF EXERCISE TOLERANCE TESTS COMPARING BASELINE TO 6 MONTHS AFTER SURGERY*

Exercise Tolerance Test	Baseline	6-Month Postop
Duration (min)	5.6 ± 2.4†	7.4 ± 2.6††
SBP × HR × (10^3 mmHg/min)	14.1 ± 2.7†	15.0 ± 4.2††
ST/HR slope	7.8 ± 8.9†††	4.3 ± 2.0†††

SBP, systolic blood pressure; HR, heart rate; ST, ST segment of electrocardiogram.
* In patients receiving intramyocardial Ad_{GV} VEGF121.10 as sole therapy (mean ± SD).
† n = 6
†† n = 5
††† n = 3

demonstrated a trend in improvement in their angina classification, nitroglycerin use, and some exercise parameters; these changes were persistent at 6 months. Should similar observations be made in large-scale controlled trials, given the 1- to 2-week period of transgene expression characteristic of Ad-mediated gene transfer, these findings suggest that the putative angiogenic effects of this therapy could persist well beyond the expected interval of VEGF angiogen expression.

Systemic Parameters

There was no evidence of deterioration of liver function or any other systemic abnormalities at 6-month follow-up in any of the treated patients in either group A or group B. This observation is important because it is theoretically possible, although inconsistent with the known biology of the Ad vector, that the activity of the Ad_{GV}VEGF121.10 vector could somehow be delayed or persistent beyond known expression profiles of the vector. Although administration of the Ad_{GV}VEGF121.10 vector to the heart did induce an increase in anti-Ad neutralizing antibodies in most of the study population, as previously reported,[17] these antibody levels returned toward baseline at 6 months in most patients, suggesting that repeat administration of this therapy might be possible.

Acknowledgments

The authors thank M.L. Lessor, North Shore University Hospital, Manhasset, New York, and N. Mohamed for help in preparing this manuscript.

References

1. Rosengart TK, Patel SR, Crystal RG. Therapeutic angiogenesis: protein and gene therapy delivery strategies. J Cardiovasc Risk 1999; 8:29–40.
2. Simons M, Ware JA. Food for starving hearts. Nat Med 1996; 2:519–520.
3. Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E. Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis. Cardiovasc Res 1994; 28:1176–1179.
4. Li J, Brown LF, Hibberd MG, Grossman JD, Morgan JP, Simons M. VEGF, flk-1, and flt-1 expression in a rat myocardial infarction model of angiogenesis. Am J Physiol 1996; 270:H1803–H1811
5. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. J Cell Biochem 1991; 47:211–218.
6. Folkman J, Haudenschild C. Angiogenesis *in vitro*. Nature 1980; 288:551–556.
7. Mack CA, Patel SR, Schwarz EA, et al. Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. J Thorac Cardiovasc Surg 1998; 115:168–176.
8. Thomas KA. Vascular endothelial growth factor, a potent and selective angiogenic agent. J Biol Chem 1996; 271:603–606.
9. Losordo DW, Vale PR, Symes JF, et al. Gene therapy for myocardial angiogenesis: initial clinical results with direct myocardial injection of phVEGF165 as sole therapy for myocardial ischemia. Circulation 1998; 98:2800–2804.
10. Mack CA, Magovern CJ, Budenbender KT, et al. Salvage angiogenesis induced by adenovirus-mediated gene transfer of vascular endothelial growth factor protects against ischemic vascular occlusion. J Vasc Surg 1998; 27:699–709.
11. Magovern CJ, Mack CA, Zhang J, et al. Direct *in vivo* gene transfer to canine myocardium using a replication-deficient adenovirus vector. Ann Thorac Surg 1996; 62:425–434.
12. Muhlhauser J, Merrill MJ, Pili R, et al. VEGF165 expressed by a replication-deficient recombinant adenovirus vector induces angiogenesis *in vivo*. Circ Res 1995; 77:1077–1086.
13. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. Mol Cell 1998; 2:549–558.
14. Rosengart TK, Lee LY, Patel SR, et al. Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing the VEGF121 cDNA to individuals with clinically significant severe coronary artery disease. Circulation 1999; 100:468–474.
15. Rosenfeld MA, Yoshimura K, Trapnell BC, et al. *In vivo* transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. Cell 1992; 68:143–155.
16. Mack CA, Song WR, Carpenter H, et al. Circumvention of anti-adenovirus neutralizing immunity by administration of an adenoviral vector of an alternate serotype. Hum Gene Ther 1997; 8:99–109.
17. Harvey B-G, Hackett NR, El-Sawy T, et al. Variability of human systemic humoral immune responses to adenovirus gene transfer vectors administered to different organs. J Virol 1999; 73:6729–6742.
18. Campeau L. Grading of angina pectoris [letter]. Circulation 1976; 54:522–523.
19. Okin PM, Kligfield P. Heart rate adjustment of ST segment depression and performance of the exercise electrocardiogram: a critical evaluation. J Am Coll Cardiol 1995; 25:1726–1735.

Discussion

DR. M. JUDAH FOLKMAN (Boston, Massachusetts): I think Dr. Rosengart and his colleagues should be congratulated for a path-breaking story. They have introduced a new modality for the treatment of ischemic heart disease by engineering the heart muscle to continually produce its own private angiogenic protein. The key point in the manuscript is that the angiogenic protein does not

appear to spill over into the main circulation, the procedure is safe, with few if any side effects, and myocardial function is improved.

Dr. Rosengart has more than a year's experience with this very new principle. And this puts him in a small club of pioneers, which includes, for example, Jeffrey Isner at St. Elizabeth Hospital in Boston, who has treated 20 patients with injection into the myocardium of naked DNA for an angiogenic protein VEGF, as used here, and these patients had no other surgical options. Yet a year later, their scans still show persistent new collateral vessels, and half have become angina-free. A third pioneer in this club is Michael Simons at Beth Israel Hospital in Boston, who injects an angiogenic protein which is different, FGF, into the myocardium through a cardiac catheter in the arm, using also sustained release polymers.

It is too early to say whether therapeutic angiogenesis of the heart will be added to coronary artery bypass surgery or will be added to angioplasty or will ever be used as first-line therapy. And Dr. Rosengart has been very understated and guarded in his presentation of this nice data.

I think what can be said is that this advance emerged unpredicted from a broad field of angiogenesis research which began 30 years ago in a pediatric surgery laboratory in a children's hospital in an attempt to study tumor angiogenesis, a grant supported by the Cancer Institute.

From this field has come another potential therapy for ischemic heart disease reported just this week in the journal *Circulation*—the demonstration in aortas of mice that atherosclerotic plaque growth is angiogenesis-dependent, and that treatment with a naturally occurring angiogenesis inhibitor endostatin suppresses plaque growth by 85%. This is the work of Karen Molton, a cardiologist in our lab.

So this new finding is not yet ready for clinical application. But if and when it is, it suggests the possibility of a two-pronged attack on ischemic heart disease. In addition to Dr. Rosengart's elegant work, there may be not only a way to stimulate blood vessels as he has shown in the heart, but also to turn off angiogenesis in plaque growth.

PRESENTER DR. TODD K. ROSENGART (New York, New York): Thank you, Dr. Folkman. And please allow me to thank you for your pioneering work that literally has allowed the creation of this entire new field.

DR. THOMAS C. MOORE (Torrance, California): Mr. President, when I approached you early this morning about discussing this important paper and you directed me to our Recorder, Dr. Brennan, it did not occur to me that I would not be the first-listed discussant. When the first-listed discussant arose and turned out to be Judah Folkman, father of angiogenesis, I was delighted—nonetheless, he is a difficult act to follow. In his acceptance last year of the 1998 Flance-Karl Award of this Association, he spoke of some of the interesting, new, and expanding observations relating to "therapeutic" and induced "good" angiogenesis which had been stimulated by his pioneering and most impressive work over the years on "bad" (cancer-related) angiogenesis.

At a national meeting in October 1995, I had the opportunity of discussing with Judah the possible, indeed likely, involvement of a "spontaneous" hypoxia-triggered good angiogenesis relating to up-regulation the VEGF family of molecules, genes, and receptors (including the BB dimer of PDGF) to account for the remarkable (almost miraculous) salvage of gut and life associated with the use of the laparotomy "patch, drain, and wait" (PD&W) approach to the surgical management of perforated necrotizing enterocolitis (NEC) and midgut volvulus (MGV) with extensive ischemia/necrosis in the newborn, an approach which I initiated in 1982 and which I have used and reported upon multiple times since (*Pediatr Surg Int* 1989;4:110–3, 1991;6:313–7, 1997;12:208–10).

Early in this experience, in the 7- to 10-day period following the PD&W operation, reoperation in two cases encountered an unforgettable massive florid hypervascularity "angiogenesis" which led to exsanguination in one case and almost in the other—good angiogenesis if the surgeon can be persuaded not to mess with it in this most critical of periods.

This type of good angiogenesis is spontaneous and hypoxia-associated rather than induced and therapeutic as in the authors' report here. Have the authors looked at hypoxia-associated levels of VEGF family of molecules in their clinical and experimental models of myocardial ischemia in comparison with normal and nonischemic myocardium, as well as after their therapeutic and induced VEGF-mediated good angiogenesis?

The laparotomy PD&W approach to severely ischemia/necrotic gut which I have initiated and which appears to be associated with a marked hypoxia-triggered elevation in the level of spontaneous good angiogenesis/hypervascularity involves a quick midline linea alba in-and-out laparotomy with extensive Penrose drain drainage of the entire peritoneal cavity, with drains from the undersurfaces of both diaphragms running down in a serpentine manner to stab wound exit sites in both lower quadrants of the abdomen and with loops into the pelvis. A monitoring gastronomy, TPN, and broad-spectrum antibiotics complete the initial undertaking. Stoma bags at Penrose drain exit sites capture fecal fistulas and function as *de facto* enterostomies. The peritoneal cavity is rapidly obliterated by adhesions and spontaneous good angiogenesis—no peritoneal cavity, no peritonitis! Spontaneous autoanastomosis occurs in approximately 70% of the cases and no second operation is needed as the Penrose drainage dries up and anal passage of fecal material resumes. With adherence to the simple but effective formula of resect no gut and do no enterostomies, both life and gut may be saved—and likely with many thanks to the good angiogenesis of the basic and hypoxia-triggered spontaneous type, which the authors have described today for the salvation of ischemia-threatened myocardium.

Have the authors looked at baseline (spontaneous rather than induced) levels of VEGF and other upregulated molecules, genes, and receptors such as serotonin, substance P, the BB-dimer of PDGF and others in their experimental acute and chronic models of myocardial ischemia, and does their induced therapeutic angiogenesis speed up or augment this baseline level, and to what degree? In other words, the question is, are you with your approach simply augmenting or speeding up a process which is sort of chugging along and not doing the job quite well enough?

In Dr. Folkman's discussion, he mentioned the exciting work of the Jeffrey Isner group in Boston with induced/therapeutic angiogenesis. In an important 1997 report by this group (*Science* 1997; 175:964–7), putative endothelial cell progenitors or angioblasts (CD34-positive mononuclear blood cells) were isolated from human peripheral blood by means of magnetic beads coated with antibody to CD34. On *in vitro* culture, these cells differentiated into endothelial cells. They confirmed an endothelial cell-like phenotype of these cells by documenting expression of ecNOS and the two VEGF receptors (Flk-1 and KDR). In animal models of ischemia, these cells homed to areas of ischemia and were incorporated into sites of active angiogenesis. These findings suggested to them that endothelial cell progenitors of this type may be useful

for augmenting collateral vessel growth to ischemic tissues (therapeutic angiogenesis). Have the authors today considered this approach or others as an addition to augmenting their exciting clinical findings?

DR. ROSENGART: Thank you very much, Dr. Moore. I would say that as Dr. Folkman has taught us, angiogenesis is really a fundamental biological process. And I think one of the reasons why we are so hopeful that therapeutic angiogenesis holds promise is that we are simply taking the native angiogenic process and augmenting it, just as we do with many of our current therapies for a number of disease states.

Certainly we and a large number of other investigators have seen the up-regulation of growth factors and receptors in the ischemic state. Again I think this highlights the point that what we are doing is simply taking the natural process and augmenting it.

I think there are a number of mediators that can be utilized. We have utilized VEGF. But certainly there are a large number of trials ongoing right now looking at different mediators to induce this strategy.

DR. ANTHONY A. MEYER (Chapel Hill, North Carolina): I enjoyed the presentation and have questions about two things. I know you looked at neutralizing antibody against the adenovirus. Did you look at neutralizing antibody to VEGF which may have been induced because of the proximity? Also, viral infections known to introduce an autoimmune response—did you look at any evidence of autoimmune response to myocardium after the injection?

DR. ROSENGART: We have thought about looking at immune responses to the VEGF *per se*. This is human VEGF. And our thinking going into these studies was that in fact it would not be immunogenic. But that is an analysis we intend to do and do have samples to perform.

In terms of your second question, we did a large number of studies in animal models, but certainly that was not equivalent to the human experience. We were admonished that it was likely that adenovirus administration to the heart was going to be a catastrophic event in terms of myocarditis.

In the animal models, again, although it did not approximate the human, there was no evidence of inflammation or micronecrosis. In the human studies we looked very, very carefully at CPK levels, motion abnormalities by echocardiogram, as well as electrocardiographic changes, and in none of the patients—and now we have treated over 30 patients—in none of these instances have we seen any evidence of myocarditis, either in patients with high preoperative antibody levels or low levels. Thirty patients still remains a small study, but certainly that has been very encouraging to us.

DR. LARRY R. KAISER (Philadelphia, Pennsylvania): I enjoyed the paper very much. I have a couple of specific questions for you regarding some of the techniques.

You have chosen to use an adenoviral vector. And we know there are some built-in limitations to adenoviral vectors, especially an adenoviral vector that is replication-deficient. This was a phase I toxicity trial. Did you ever reach your maximum tolerated dose? Or was that ever the intent?

We have talked a lot about efficacy, but of course, efficacy is not really the endpoint of a phase I trial. I noticed that in your sole therapy group, I believe you used 10^9 pfu. Is that a dose that you think will be efficacious? Or is that the dose that the FDA allowed to you use and have you subsequently used a higher dose of vector?

Also, would you comment on the possibility of using a replication-competent factor, because the efficiency of transduction here likely is not very good. We know that when we try to transduce tumors, at best we get about 10% of the cells transduced. Do you have any idea of the efficiency of transduction with your particular vector? And you might comment on how you might improve that.

DR. ROSENGART: In animal studies, a dose response is demonstrable over a viral particle administration range of 4 to 6 logs. We have not seen a dose response in the human trials, although numbers have been very, very small. Our dosing was based on therapeutic effects in animal studies. Toxicity was demonstrated only in small-animal models at several logs above that needed for efficacy.

In terms of our use of the replication-competent virus, we would be hesitant to do that, certainly. One of our feelings is that many of the preclinical studies in which inflammation was seen may, in fact, be related to the number of replication-competent particles. We think one of the reasons why we do not see inflammation is that we have extremely low titers of replication-competent particles.

In terms of the extent of transfection, at least in myocardium in animals, in the tissues that we have looked at we have high levels of transfection and were able to produce VEGF expression on the level of 1 ng per mg of protein, certainly a level that we think is very adequate to induce angiogenesis.

DR. LAZAR J. GREENFIELD (Ann Arbor, Michigan): In terms of functional significance of the collateral perfusion, were you able to demonstrate any change on cardiac scans?

DR. ROSENGART: We have looked at perfusion scans, echocardiograms, and actually wall motion on the perfusion scans. There is evidence of improvement of wall motion in these studies. It is difficult to assess these small changes in the clinical setting, so we are hesitant to present that data until further analysis. In animal studies, using an ameroid constrictor in pigs, in fact, we were able to induce very significant improvement in function, regional wall motion with this therapy.

Gene Therapy for Myocardial Angiogenesis

Initial Clinical Results With Direct Myocardial Injection of phVEGF$_{165}$ as Sole Therapy for Myocardial Ischemia

Douglas W. Losordo, MD; Peter R. Vale, MD; James F. Symes, MD; Cheryl H. Dunnington, MS; Darryl D. Esakof, MD; Michael Maysky, MD; Alan B. Ashare, MD; Kishor Lathi, MD; Jeffrey M. Isner, MD

Background—We initiated a phase 1 clinical study to determine the safety and bioactivity of direct myocardial gene transfer of vascular endothelial growth factor (VEGF) as sole therapy for patients with symptomatic myocardial ischemia.

Methods and Results—VEGF gene transfer (GTx) was performed in 5 patients (all male, ages 53 to 71) who had failed conventional therapy; these men had angina (determined by angiographically documented coronary artery disease). Naked plasmid DNA encoding VEGF (phVEGF$_{165}$) was injected directly into the ischemic myocardium via a mini left anterior thoracotomy. Injections caused no changes in heart rate (pre-GTx=75±15/min versus post-GTx=80±16/min, P=NS), systolic BP (114±7 versus 118±7 mm Hg, P=NS), or diastolic BP (57±2 versus 59±2 mm Hg, P=NS). Ventricular arrhythmias were limited to single unifocal premature beats at the moment of injection. Serial ECGs showed no evidence of new myocardial infarction in any patient. Intraoperative blood loss was 0 to 50 cm^3, and total chest tube drainage was 110 to 395 cm^3. Postoperative cardiac output fell transiently but increased within 24 hours (preanesthesia=4.8±0.4 versus postanesthesia=4.1±0.3 versus 24 hours postoperative=6.3±0.8, P=0.02). Time to extubation after closure was 18.4±1.4 minutes; average postoperative hospital stay was 3.8 days. All patients had significant reduction in angina (nitroglycerin [NTG] use=53.9±10.0/wk pre-GTx versus 9.8±6.9/wk post-GTx, P<0.03). Postoperative left ventricular ejection fraction (LVEF) was either unchanged (n=3) or improved (n=2, mean increase in LVEF=5%). Objective evidence of reduced ischemia was documented using dobutamine single photon emission computed tomography (SPECT)-sestamibi imaging in all patients. Coronary angiography showed improved Rentrop score in 5 of 5 patients.

Conclusions—This initial experience with naked gene transfer as sole therapy for myocardial ischemia suggests that direct myocardial injection of naked plasmid DNA, via a minimally invasive chest wall incision, is safe and may lead to reduced symptoms and improved myocardial perfusion in selected patients with chronic myocardial ischemia. (***Circulation.*** **1998;98:2800-2804.)**

Key Words: angiogenesis ■ ischemia ■ myocardium

Intramuscular transfection of genes encoding angiogenic cytokines[1] may constitute an alternative treatment strategy for patients with severe myocardial ischemia. This strategy is designed to promote the development of supplemental collateral blood vessels that will constitute endogenous bypass conduits around occluded native arteries, a strategy termed "therapeutic angiogenesis."[2]

This study describes the initial clinical experience with myocardial gene transfer as sole therapy for refractory angina pectoris. Five patients with chronic, severe angina underwent direct myocardial gene transfer of naked DNA encoding vascular endothelial growth factor (VEGF). There were no operative complications. All patients experienced marked symptomatic improvement and/or objective evidence of improved myocardial perfusion. This preliminary clinical experience suggests that therapeutic angiogenesis represents a potentially useful strategy for patients with coronary artery disease.

Methods

Patients

Patients were eligible for intramyocardial gene therapy if they had functional class 3 or 4 exertional angina, refractory to maximum medical therapy, areas of viable but underperfused myocardium, and multivessel occlusive coronary artery disease. Subjects were excluded if they had any of the following: a successful revascularize-

Received September 8, 1998; revision received October 28, 1998; accepted November 9, 1998.

From the Departments of Medicine, Biomedical Research, Surgery, and Anesthesiology, St. Elizabeth's Medical Center, Tufts University School of Medicine, Boston, Mass.

Correspondence to Jeffrey M. Isner, MD, St. Elizabeth's Medical Center, 736 Cambridge St., Boston, MA 02135.

Circulation is available at http://www.circulationaha.org

TABLE 1. Demographic and Clinical Data

Patient	Age	DM	Prior Revascularization	CCS FC Pre-	CCS FC Post-	Medications
1	67	0	CABG 1988; PTCA 1997	4	1	ASA, BB, N
2	69	+	CABG 1992, 1997	4	2	ASA, BB, CCB, D, N
3	53	0	PTCA 1989; CABG 1991, 1992	4	2	ASA, CCB, N
4	71	0	CABG 1982, 1984; PTCA 1992	4	1	ASA, BB, D, N
5	59	+	CABG 1994; PTCA 1996, 1997	4	2	ACE, ASA, BB, D, N

Pre- and Post- refer, respectively, to status of gene therapy.

ACE indicates angiotensin converting enzyme inhibitor; ASA, aspirin; BB, beta-blocker; CABG, coronary artery bypass graft; CCB, calcium channel blocker; CCS, Canadian Cardiovascular Study; D, diuretic; DM, diabetes; FC, functional class; N, nitrates; PTCA, percutaneous coronary angioplasty including balloon angioplasty, stent, directional and rotational atherectomy.

tion within the previous 6 months, cancer, retinopathy, or an ejection fraction (EF) <20%.

Plasmid DNA ($phVEGF_{165}$)

All patients received eukaryotic expression vector encoding the 165-amino acid isoform of the human VEGF gene (previously described)[3,4] transcriptionally regulated by the cytomegalovirus promoter/enhancer ($phVEGF_{165}$).[5,6]

Myocardial $phVEGF_{165}$ Transfer

Plasmid DNA (125 μg) was administered by direct myocardial injection in 4 aliquots of 2.0 mL each via a mini-thoracotomy to the anterolateral left ventricular free wall. Continuous transesophageal echocardiographic monitoring was performed throughout the procedure. Patients were extubated in the operating room and monitored according to the protocol used for minimally invasive CABG.

SPECT Myocardial Perfusion Study

Subjects underwent a dobutamine single photon emission computed tomography (SPECT)-sestamibi study <2 weeks before gene transfer, with the use of dobutamine infusion up to 40 $\mu g \cdot kg^{-1} \cdot min^{-1}$. The acquisition of the poststress SPECT image began 10 minutes after the end of the stress period. Redistribution images were recorded either before or at least 4 hours after stress with the subject at rest. Redistribution and reinjection data were reconstructed in short-axis, vertical, and longitudinal long-axis views for analysis. With the use of the 13-segment model, viability and perfusion scores were assigned to each segment on the basis of the results of the nuclear scan. Perfusion was recorded as normal or abnormal. Segments were visually characterized as fixed, partially reversible, or totally reversible. On days 30 and 60, subjects underwent repeat nuclear perfusion testing using the identical stress protocol and isotope used at baseline.

Coronary Angiography

Patients underwent diagnostic angiography <1 month before and 60 days after gene transfer. All angiograms were interpreted by a reviewer blinded to the patient's name, date of study, and sequence of study (ie, pre- versus posttreatment). Collaterals were graded[7] as absent (0); filling of side-branches of a target-occluded epicardial coronary artery via collaterals without visualization of the epicardial coronary artery itself (1+); partial filling of the epicardial segment via collateral arteries (2+); and complete filling of the epicardial segment (3+). Each pair of films (baseline and follow-up) was scored independently.

Statistical Analysis

Data are reported as mean±SEM. Comparisons between paired variables were performed using a Student t test with a significance level of $P<0.05$.

Results

Patients

Demographic and clinical data for the 5 men (aged 63.8±3.4 years) treated with $phVEGF_{165}$ are shown in Table 1.

Perioperative Course

All patients underwent successful myocardial gene transfer. Mean operative time was 101.6±8.9 minutes. Patients were extubated 18.4±1.4 minutes postoperatively. Injections caused no changes in heart rate (75±15/min versus 80±16/min), systolic blood pressure (114±7 versus 118±7 mm Hg), or diastolic BP (57±2 versus 59±2 mm Hg). Ventricular arrhythmias were limited to unifocal extrasystolic beats (maximum n=5) at the moment of injection. Postoperative Cardiac output fell transiently but increased within 24 hours (preanesthesia=4.8±0.4 versus postanesthesia=4.1±0.3 versus 24 hours postoperative=6.3±0.8, $P=0.02$). Serial ECGs showed no evidence of myocardial infarction in any patient; no patient had an increase in creatine kinase isoenzyme above normal limits. Intraoperative blood loss was 5 to 50 cm^3, and total chest tube drainage was 110 to 395 cm^3. There were no major perioperative complications. Postoperative LVEF was either unchanged (n=3) or improved (n=2, mean increase in LVEF=5%). All patients were discharged on postoperative day 4 except patient 2 who was discharged on postoperative day 3.

Change in Clinical Status

All 5 patients experienced a decrease in anginal frequency and severity (Table 1). There was no change in the anginal

TABLE 2. Perfusion Scan Results

	Number of Segments								
	Normal Perfusion			Reversible Defect			Fixed Defect		
Patient	Base	D30	D60	Base	D30	D60	Base	D30	D60
1	8	9	9	3	3	3	2	1	1
2	9	9	10	2	4	3	2	0	0
3	5	6	6	5	6	6	3	1	1
4	5	7	8	5	4	3	3	2	2
5	3	6	7	8	5	4	2	2	2

Base indicates baseline; D30, 30-day follow-up study; D60, 60-day follow-up study.

TABLE 3. Angiographic Results

Patient	Extramural Vessel	Rentrop Score	
		Pre-GTx	Post-GTx
1	RCA via LAD/Diag via SVG	1	2
2	OMB via SVG	1	2
	PDA via LAD via LIMA	1	2
3	RCA via LCX	0	3
	Diag via LAD via LIMA	1	3
4	Diag via LAD via LIMA	0	1
	RCA via LCX and Septal	1	2
5	Diag via LAD via LIMA	0	1

RCA indicates right coronary artery; LAD, left anterior descending; Diag, diagonal; SVG, saphenous vein graft; OMB, obtuse marginal branch; PDA, posterior descending artery; LIMA, left internal mammary artery; and LCX, left circumflex.

pattern in any patient up to 10 days post–gene transfer. All patients began to experience a reduction in angina between 10 and 30 days after gene transfer. Angina was completely abolished in 2 patients (patients 1 and 4); patient 5, who has previously experienced daily angina, had only 2 episodes of angina between the day 30 and day 60 follow-up visits. Patients 2 and 3 continued to experience occasional angina but with reduced frequency and at much higher levels of activity. Nitroglycerin (NTG) use for the group of 5 patients decreased from 7.7 ± 1.4 to 1.4 ± 1.0 tablets per day by 60 days post–gene transfer ($P<0.05$). Brief synopses of the clinical courses of these 5 patients are provided below.

Patient 1, a 67-year-old man, experienced daily angina induced by mild activity requiring an average of 8 tablets NTG/d. All native vessels and 3 of 4 bypass grafts were occluded. Several institutions had advised the patient that the small caliber of his remaining native vessels precluded repeat CABG. Beginning 21 days after gene transfer, the patient experienced a decrease in the frequency and severity of his angina. By postoperative day 60, the patient was no longer experiencing angina and was no longer requiring NTG. He was able to engage in activities, such as swimming, which were previously impossible because of anginal pain.

Patient 2, a 69-year-old man, experienced daily angina precipitated by activity such as walking 10 yards; for several months he had been taking 12 tablets NTG/d. A vein graft to the left obtuse marginal (LOM) was occluded, and a diffusely diseased vein graft to a diagonal branch of the left anterior descending (LAD) coronary artery was not amenable to percutaneous revascularization. Additional surgery was not feasible because of poor target vessels. For 3 weeks after gene transfer, his symptoms remained unchanged. The patient then began to notice a decrease in NTG consumption accompanied by the ability to increase his level of activity. By day 60, the patient was able to exercise on the bicycle at his local gymnasium for up to 30 minutes. The patient's NTG requirement decreased to a maximum of 2 tablets/d for occasional episodes of mild angina.

Patient 3, a 53-year-old man, experienced daily angina induced by walking ≤50 yards and used 6 NTG tablets/d. All native vessels were occluded; grafts to the LAD and right coronary artery (RCA) were patent, whereas an LOM graft was occluded. Percutaneous revascularization was not possible and a third bypass operation for single vessel bypass to a small-caliber target vessel was not feasible. The patient

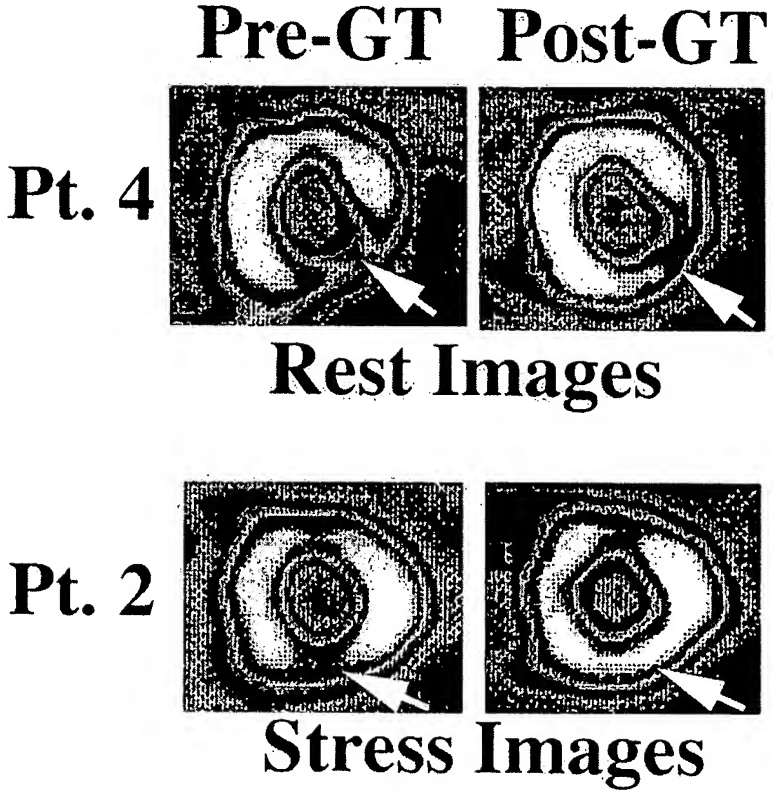


Figure 1. SPECT-sestamibi perfusion imaging. Top, Example of improvement in a "fixed" defect (perfusion abnormality on resting image). In patient (Pt.) 4, a moderate area of decreased perfusion is seen in the infero-lateral wall (arrow) before gene therapy. After gene therapy, perfusion is improved. Bottom, Example of improvement in an area of ischemia. In Pt. 2, a small zone of decreased perfusion is seen in the inferior wall (arrow) before treatment. After treatment, the matching scan shows no evidence of this perfusion defect while a zone of ischemia on the anterior (opposite) wall persists.

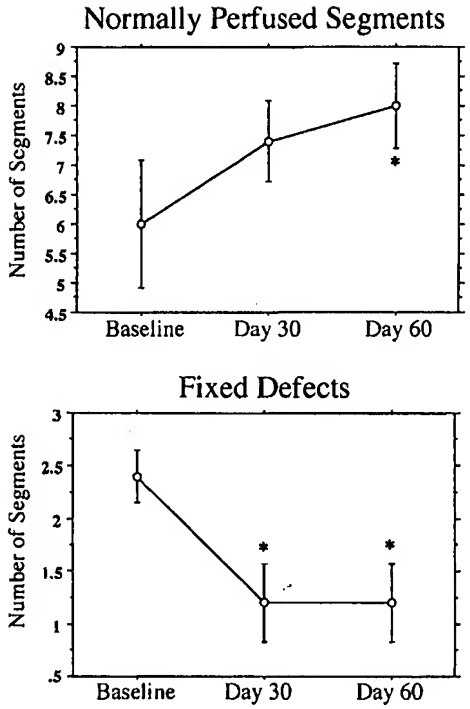


Figure 2. SPECT-sestamibi perfusion imaging: summary of findings in 5 patients. Short-axis views were divided into a total of 13 segments and graded as normal (no perfusion defect), reversible (perfusion defect during stress that partially or completely reversed at rest), or fixed (perfusion defect during stress that persists at rest). Values represent mean±SEM for all 5 patients at baseline, 30 days, and 60 days post-gene therapy. *$P<0.05$ compared with baseline.

experienced no change in anginal symptoms until postoperative week 2, when he noticed an increase in the level of activity required to induce angina. At that time, he was able to perform activities (eg, planting in his garden) that he had not previously engaged in for several months; NTG use decreased to 5 tablets per week. By 60 days after gene transfer, he was able to walk up to one-half of a mile without experiencing angina.

Patient 4, a 71-year-old man, complained of daily angina precipitated by walking <100 yards. All native vessels and grafts to the RCA and LOM were occluded. Percutaneous revascularization was not possible and repeat surgery was not feasible because of small-caliber target vessels. Beginning on postoperative day 10, the patient noted increased exercise capacity accompanied by decreased NTG use. By day 30 follow-up, the patient was requiring no NTG and had returned to his 5 hour per day position doing maintenance for his church. Between days 30 and 60, the patient developed dyspnea, associated with inadvertent discontinuation of his daily diuretic (furosemide, 80 mg). After resumption of his diuretic, his symptoms resolved and he resumed his increased activity level without anginal symptoms, dyspnea, or NTG use.

Patient 5, a 59-year-old man with daily angina precipitated by walking 10 to 20 yards, also required continuous oxygen because of severe chronic obstructive pulmonary disease. He had been recently hospitalized for several months because of intractable angina requiring intravenous NTG. All native vessels and grafts to RCA and diagonal branch of the LAD were occluded. Percutaneous revascularization was not possible and a third bypass operation was not feasible because of poor distal vessels. By postoperative day 30, the patient noted that he was experiencing no angina and was able to walk distances of up to 500 yards. Additionally, he found that his use of supplemental oxygen had decreased. At day 60 follow-up, he reported a total of 2 anginal episodes in the previous month, each of which was resolved with a single NTG tablet.

SPECT-Sestamibi Perfusion Imaging

All patients had improvement in myocardial perfusion, revealed by comparison between pre- and posttreatment (Figure 1) SPECT-sestamibi imaging (Table 2). The mean number of normally perfused segments per patient increased from 6.0±1.1 before gene transfer to 8.0±0.7 ($P<0.05$) at day 60 after gene transfer (Figure 2). This was accompanied by a decrease in the mean number of irreversibly ischemic segments from 2.4±0.2 to 1.2±0.4 ($P<0.05$) at day 60 follow-up examination (Figure 2).

Coronary Angiography

Selective coronary angiography was performed before and 59.8±1.5 days after gene transfer (Table 3). Angiographic evidence for improved collateral flow into ischemic areas of the myocardium was observed in all 5 patients. The evidence of new collateral vessels consisted of improved filling of 4 previously identified vessels as well as the development of collaterals to 3 vessels which previously had no collateral filling. In 2 patients, there was improvement by a single Rentrop grade in one vessel territory; the other 3 patients demonstrated improvement in 2 territories by 1 to 3 Rentrop grades.

Discussion

The finding that VEGF could be used to achieve angiogenesis that was therapeutic was first demonstrated by Takeshita et al,[2] who administered rhVEGF as a single intra-arterial bolus to rabbits with unilateral hindlimb ischemia. Similar findings with rhVEGF administration in canine[8] and porcine[9] models of myocardial ischemia were published shortly thereafter.

Gene transfer constitutes an alternative strategy for accomplishing therapeutic angiogenesis in patients with limb and myocardial ischemia. In VEGF, this is a particularly appealing strategy because the VEGF gene encodes a signal sequence which permits the protein to be naturally secreted from intact cells.[4] Previous studies from our laboratory[10,11] indicated that arterial gene transfer of cDNA encoding for a secreted protein could yield meaningful biological outcomes despite a low transfection efficiency. Indeed, preclinical animal studies established the feasibility of achieving therapeutic angiogenesis after site-specific gene transfer of naked DNA encoding $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$.[12] Subsequent clinical experience documented histological and angiographic evidence of $phVEGF_{165}$-induced neovascularization in patients with critical limb ischemia.[5,6] These findings established proof of principle for the concept that the angiogenic activity of VEGF is sufficiently potent to achieve therapeutic benefit.

The present study provides the first evidence for a favorable clinical effect of direct myocardial injection of naked plasmid DNA encoding for VEGF. Each patient experienced a reduction in anginal symptoms and nitrate use, and there is objective evidence for reduced ischemia by perfusion imaging. Because each patient enrolled in this study had long-standing, stable, severe angina, the change in clinical status observed for these 5 patients is unlikely to represent random chance. In contrast to work recently reported by Schumacher et al,[13] in which administration of fibroblast growth factor-1 (FGF-1) was combined with conventional surgical revascularization,[13] the present study used VEGF gene transfer as the sole therapeutic intervention.

This early experience, although encouraging from the standpoint of therapeutic angiogenesis and gene therapy, leaves several issues unresolved. Optimizing the anatomic site, number, and dose of intramyocardial injections will require further investigation. The FDA, Recombinant Advisory Committee of the NIH, and St. Elizabeth Medical Center Human Investigation Research and Institutional Biosafety Committees all concurred that the strategy of gene therapy alone administered via a mini-thoracotomy would not permit randomization against placebo (untreated controls). We anticipate that incorporation of a placebo group and clinical testing of alternative dosing regimens, including multiple treatments, will be addressed on availability of a catheter-based system for reliable percutaneous myocardial gene delivery; this is currently under preclinical investigation.[14]

Furthermore, the choice of appropriate formulation or vector in the case of VEGF remains to be determined. As indicated above, rhVEGF protein has been shown to be efficacious for treatment of limb and myocardial ischemia in preclinical studies, and preliminary clinical investigation of rhVEGF[15] together with the aforementioned studies of Schumacher et al have suggested the potential usefulness of recombinant protein for therapeutic angiogenesis. The use of an adenoviral vector expressing $VEGF_{121}$ has been shown to improve myocardial perfusion and function in a swine model of myocardial ischemia[16] and is now being tested in human subjects. Likewise, alternatives to VEGF, including FGF-1,[17] FGF-2,[18] and FGF-5[19] are or will be investigated as genes or recombinant proteins in clinical trials of therapeutic angiogenesis.

References

1. Folkman J, Klagsbrun M. Angiogenic factors. *Science.* 1987;235: 442–447.
2. Takeshita S, Zheng LP, Brogi E, Kearney M, Pu LQ, Bunting S, Ferrara N, Symes JF, Isner JM. Therapeutic angiogenesis: a single intra-arterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hindlimb model. *J Clin Invest.* 1994;93:662–670.
3. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science.* 1989;246:1306–1309.
4. Tischer E, Mitchell R, Hartmann T, Silva M, Gospodarowicz D, Fiddes J, Abraham J. The human gene for vascular endothelial growth factor: multiple protein forms are encoded through alternative exon splicing. *J Biol Chem.* 1991;266:11947–11954.
5. Isner JM, Pieczek A, Schainfeld R, Blair R, Haley L, Asahara T, Rosenfield K, Razvi S, Walsh K, Symes J. Clinical evidence of angiogenesis following arterial gene transfer of $phVEGF_{165}$. *Lancet.* 1996;348: 370–374.
6. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM. Constitutive expression of $phVEGF_{165}$ following intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation.* 1998;97:1114–1123.
7. Rentrop KP, Cohen M, Blanke H, Phillips RA. Changes in coronary collateral filling immediately after controlled coronary artery occlusion by an angioplasty balloon in human subjects. *J Am Coll Cardiol.* 1985; 5:587–592.
8. Banai S, Jaklitsch MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, Epstein SE, Unger EF. Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation.* 1994;89:2183–2189.
9. Pearlman JD, Hibberd MG, Chuang ML, Harada K, Lopez JJ, Gladston SR, Friedman M, Sellke FW, Simons M. Magnetic resonance mapping demonstrates benefits of VEGF-induced myocardial angiogenesis. *Nature Med.* 1995;1:1085–1089.
10. Losordo DW, Pickering JG, Takeshita S, Leclerc G, Gal D, Weir L, Kearney M, Jekanowski J, Isner JM. Use of the rabbit ear artery to serially assess foreign protein secretion after site specific arterial gene transfer in vivo: evidence that anatomic identification of successful gene transfer may underestimate the potential magnitude of transgene expression. *Circulation.* 1994;89:785–792.
11. Takeshita S, Losordo DW, Kearney M, Isner JM. Time course of recombinant protein secretion following liposome-mediated gene transfer in a rabbit arterial organ culture model. *Lab Invest.* 1994;71:387–391.
12. Takeshita S, Tsurumi Y, Couffinhal T, Asahara T, Bauters C, Symes JF, Ferrara N, Isner JM. Gene transfer of naked DNA encoding for three isoforms of vascular endothelial growth factor stimulates collateral development in vivo. *Lab Invest.* 1996;75:487–502.
13. Schumacher B, Pecher P, von Specht BU, Stegmann TH. Induction of neoangiogenesis in ischemic myocardium by human growth factors: first clinical results of a new treatment of coronary heart disease. *Circulation.* 1998;97:645–650.
14. Vale PR, Losordo DW, Symes JF, Isner JM. Gene therapy for myocardial angiogenesis. *Circulation.* 1998;98(suppl I):I-322. Abstract
15. Henry TD, Rocha-Singh K, Isner JM, Kereiakes DJ, Giordano FJ, Simons M, Losordo DW, Hendel RC, Bonow RO, Rothman JM, Borbas ER, McCluskey ER. Results of intracoronary recombinant human vascular endothelial growth factor (rhVEGF) administration trial. *Circulation.* 1998;31:65A. Abstract.
16. Mack CA, Patel SR, Schwarz EA, Zanzonico P, Hahn RT, Ilercil A, Devereux RB, Goldsmith SJ, Christian TF, Sanborn TA, Kovesdi I, Itackett N, Isom OW, Crystal RG, Rosengart TK. Biologic bypass with the use of adenovirus-mediated gene transfer of the complementary deoxyribonucleic acid for vascular endothelial growth factor 121 improves myocardial perfusion and function in the ischemic porcine heart. *J Thorac Cardiovasc Surg.* 1998;115:168–176.
17. Selke FW, Jianyi L, Stamler A, Lopez JJ, Thomas KA, Simons M. Angiogenesis induced by acidic fibroblast growth factor as an alternative method of revascularization for chronic myocardial ischemia. *Surgery.* 1996;120:182–188.
18. Harada K, Grossman W, Friedman M, Edelman ER, Prasad PV, Keighley CS, Manning WJ, Selke FW, Simons M. Basic fibroblast growth factor improves myocardial function in chronically ischemic porcine hearts. *J Clin Invest.* 1994;94:623–630.
19. Giordano FJ, Ping P, McKirnan D, Nozaki S, DeMaria AN, Dillmann WH, Mathieu-Costello O, Hammond HK. Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart. *Nature Med.* 1996;2:534–539.

Gut 2000;46:136–139

Review

Gene therapy of hepatic diseases: prospects for the new millennium

As we stand on the cusp of the 21st century, the prospect of treating liver diseases by the manipulation of genetic material seems to be not merely a remote possibility, but a tangible reality. The rapid advances in biotechnology over the past few decades have afforded a unique understanding of the molecular mechanisms underlying various hepatic disorders. As a result, novel and exciting techniques have been developed for the genetic modification of hepatocytes. In this article, we will highlight advances in particular areas, and lend perspective as to their future prospects.

Strategies for hepatic gene therapy

GENE AUGMENTATION THERAPY

This strategy involves administration of a normal gene to replace a missing or dysfunctional gene product resulting from a defective gene, as has been illustrated by studies on the hereditary disorder familial hypercholesterolaemia. In this disease, a defect in the low density lipoprotein (LDL) receptor gene results in abnormal expression of the LDL receptor and consequent failure of clearance of LDL cholesterol.[1] Using an animal model of familial hypercholesterolaemia, the Watanabe heritable hyperlipidaemic (WHHL) rabbit, investigators have been able to show the successful transduction of a functional rabbit LDL gene into target hepatocytes. This resulted in a 30–40% reduction in serum cholesterol, with the recombinant LDL receptor being detectable for up to six months.[2] In clinical trials, five patients homozygous for familial hypercholesterolaemia underwent ex vivo replacement of the faulty gene.[3] This was achieved by segmental hepatic resection, preparation of hepatocyte cultures, and transduction of these cultures with a recombinant retrovirus encoding the gene for the human LDL receptor. The genetically modified cells were then transplanted into the liver using portal venous cannulation. Prolonged reductions in LDL cholesterol were seen in three of the five patients, and this procedure was remarkably free of any major side effects. This study served to demonstrate the feasibility of ex vivo therapy, although concerns remain regarding the long term efficacy of this approach. Another disadvantage is the limited availability of autologous hepatocytes. However, recent advances in the propagation of liver cells such as the introduction of temperature sensitive mutant Simian virus 40 (SV-40) T antigen mutants[4] may obviate the need for harvests of large numbers of host cells for genetic manipulation.

In vivo approaches to genetic manipulation involve the transfer of genes to target tissue by either systemic administration or direct injection. The paradigm for in vivo therapy has been the approach to ornithine transcarbamylase (OTC) deficiency. Using the sparse-fur mouse model of OTC deficiency, an intravenous injection of an adenoviral vector has been shown to produce a high level gene transfer, leading to expression of the functional enzyme.[5] Based on these experiments, a human clinical trial has recently been approved for the genetic treatment of adults with partial OTC deficiency. When available, the results of this study will provide further valuable insights into the clinical applications of this technology.*

Another recent in vivo experiment demonstrated the use of retroviral vectors to produce sustained expression of therapeutic levels of factor VIII in a neonatal mouse model of haemophilia A.[6] As it is desirable to institute gene therapy early in life, the propagation of neonatal hepatocytes represents a promising approach with clinical relevance.

REPAIR OF ABNORMAL GENES

An ideal approach to gene therapy would be to repair the defective gene. This is particularly true for dominant negative mutations where simple introduction of a normal gene product may not overcome the dysfunction caused by the continued production of an abnormal product. Such repair processes do occur naturally, but at very low frequency. Recently, a novel technique has been devised to correct a faulty gene by harnessing the repair mechanisms of the host. In this approach, a molecule is prepared composed of both DNA and RNA domains forming a chimeric RNA/DNA oligonucleotide.[7] The RNA domain of the oligonucleotide is designed to be perfectly complementary to the targeted gene except for a single base mismatch. The mismatch seems to trigger the cell's DNA repair mechanism, and induces a nucleotide substitution at the target site. In an in vitro application of this technique, chimeric RNA/DNA oligonucleotides were used to introduce successfully a point mutation into the chromosomal alkaline phosphatase gene in a human hepatoma cell line.[8] The base conversion efficiency was calculated to be approximately 40% based on amplification and cloning. This approach was extended to an in vivo setting, targeted against the rat factor IX gene.[9] The desired mutation was noted in up to 40% of target sites. However, a similar degree of success has not been duplicated outside of that laboratory. It is possible that the methods used to assess efficiency overestimate the actual figure. Nevertheless, the studies represent an important landmark in the field of genetic research.

INHIBITION OF ENDOGENOUS GENE EXPRESSION

Acquired liver disorders represent a more heterogeneous array of conditions, some of which may be amenable to genetic therapy by inhibiting the expression of various genes. Examples include infections with the hepatitis viruses where viral gene products can be blocked, and malignancies in which an oncogene or other growth regulatory genes can be inhibited. Several types of molecular agents have been developed.

*Note added in proof: A troubling development has occurred in a clinical trial of adenoviral gene therapy for ornithine transcarbamylase deficiency. The unexplained death of one of the trial patients has resulted in the indefinite suspension of the trial.

Abbreviations used in this review: AAV, adeno-associated virus; AsGPr, asialoglycoprotein receptor; BUGT, bilirubin-UDP-glucuronyltransferase; GSD, glycogen storage disease; HCV, hepatitis C virus; HSV-tk, herpes simplex virus thymidine kinase; hGAA, human acid α-glucosidase; IL, interleukin; LDL, low density lipoprotein; OTC, ornithine transcarbamylase; SV-40, Simian virus 40.

Ribozymes
Ribozymes are single stranded RNA molecules that contain a cleavage domain flanked by target binding domains on each side. These molecules can destroy target messenger RNA sequences in a catalytic manner. However, their use is limited by the susceptibility of RNA to nuclease degradation. Recently, ribozyme analogues composed of DNA (DNA ribonucleases) which are less sensitive to degradation have been developed.[10] The latter have been demonstrated in vitro to inhibit hepatitis B viral gene expression substantially.[11]

Antisense oligonucleotides
Antisense oligonucleotides have also been successfully used to block endogenous gene expression in the Peking duck and woodchuck animal models of chronic hepatitis B.[12 13] This involves preparing a short single stranded DNA segment, or a gene which will generate RNA, which is complementary to a target messenger RNA. Within the cell, the antisense nucleic acid hybridises with and specifically inhibits translation of the particular gene product. There have been reports of systemic infusion of antisense oligonucleotides resulting in substantial liver uptake.[14] In addition, targeted delivery of inhibitory oligonucleotides has been demonstrated in vitro raising the possibility of decreasing potential side effects at other non-hepatic sites. Such targeted antisense nucleotides directed against the 5'-non-translated region of hepatitis C virus (HCV) were shown to inhibit HCV protein synthesis effectively.[15] Targeted inhibition of hepatitis B viral gene expression has also been demonstrated in vitro and in vivo. However, the low efficiency and possible immune response to the carriers are potential drawbacks.

VIRAL ENZYME PRODRUG THERAPY
A gene therapy strategy for the treatment of primary and metastatic liver cancer has been developed in which tumour cells are transduced with a non-mammalian "suicide" gene which can convert a non-toxic prodrug into a chemotherapeutic agent exclusively within the target malignant cells.[16] As a result, the systemic toxicity often associated with chemotherapy may be avoided. For example, the herpes simplex virus thymidine kinase (HSV-tk) gene which phosphorylates the synthetic guanine analogue, ganciclovir, into a toxic metabolite[17] and cytosine deaminase which converts 5-fluorocytosine into the chemotherapeutic agent 5-fluorouracil[16] have been adapted for this purpose. The genes for these enzymes when introduced into malignant cells have been shown to generate sufficient toxin to destroy those cells selectively. In addition, a bystander effect has been shown to occur in which surrounding tumour cells are also affected. In this way, even cells not transfected with the gene can be destroyed. Nevertheless, the prospect of eliminating every malignant cell will depend on high efficiency of transfection.

Co-delivery of genes encoding antitumour cytokines (e.g. interleukin-2 (IL-2) and granulocyte–macrophage colony stimulating factor) in an attempt to stimulate antitumour immunity[18] has also been developed. One recent study using this so-called "cancer vaccine" approach used a murine model transfected by both HSV-tk and IL-2. Major tumour regression was noted and was associated with the apparent development of systemic antitumoral immunity. Further studies will be required to determine whether this approach will have clinical applications.

Methods of gene delivery to the liver

The importance to successful gene therapy of developing effective gene delivery systems cannot be overstated. An ideal vector would be able to deliver genetic material efficiently and specifically, and would result in high level, properly regulated and prolonged expression. This vector should be non-toxic, non-immunogenic, and have a broad host range.

VIRAL VECTORS
Viruses have many advantages as vehicles for the delivery of genes, as this is their normal function. There have been a number of recent advances in this area.

Retroviral vectors
The ability to integrate into the host genome[19] and to be transmitted to progeny cells has made retroviral vectors very popular. Their obvious advantages of efficient and specific gene delivery are, however, offset by certain drawbacks. The most important of these is that retroviruses are unable to infect non-dividing cells. This has led to the administration of substances to stimulate hepatocyte proliferation[20] and the development of lentiviral vectors.[21] Lentiviruses are a class of complex retroviruses, the best known of which is HIV-1. They are able to infect non-dividing terminally differentiated tissue such as the liver while retaining the other advantages of retroviral vectors. Lentiviruses seem to have an excellent biosafety profile. However, as there is no animal model for HIV-1 infection, the efficacy and safety of these vectors can only be tested in clinical trials. Another potential drawback associated with both retroviral and lentiviral vectors is their random integration into the recipient genome. This has led to concerns that this integration may activate oncogenes or inactivate tumour suppressor genes.

Adenoviral vectors
Adenoviruses 2 and 5 (subgroup C) are commonly used as gene vectors. Recombinant adenoviruses may be generated in high titres, and these viruses localise predominantly in the liver after systemic administration.[22] The transfer of genetic material is usually quick and efficient. Despite these obvious advantages, the use of adenoviral vectors has been limited by several factors. Owing to the episomal nature of the virus, transgene expression is transient.[23] The prototype viruses were immunogenic, resulting in a strong host response.[24] Several strategies to attenuate the host's immune response have been studied. These include the use of transient immunosuppression by agents such as tacrolimus (FK 506)[25] and the modification of the virus by deleting certain genes.[26] This so-called "gutless" adenovirus is, however, still able to evoke an antiviral immune response. Another strategy is the enhancement of immunomodulatory genes produced by the adenovirus itself.[27] If these immunomodulatory genes are enhanced, allogeneic cell rejection has been shown to be greatly reduced. Another important recent study has focused on the treatment of glycogen storage disease II (GSD-II) using a modified adenovirus vector encoding human acid α-glucosidase (hGAA).[28] In a knockout mouse model of GSD II, hepatic transduction with the adenoviral vector resulted in high level secretion of the precursor form of the hGAA enzyme into the circulation, with consequent peripheral uptake by skeletal muscle and decreased glycogen accumulation in affected muscles. This approach is diametrically different to previous studies in this area, in that it has targeted the liver, rather than affected fibroblasts. If confirmed by other researchers, these findings offer exciting therapeutic possibilities for these otherwise incurable metabolic disorders.

Adeno-associated viral vectors
Adeno-associated virus (AAV) is a human non-pathogenic replication-defective parvovirus. One of the major potential

advantages of this system is the property of site specific integration in the presence of a *rep* gene product. The wild type viral DNA can integrate preferentially into human chromosome 19. However, recombinant AAV integrates randomly when the *rep* gene is absent. Other advantages of recombinant AAV vector system are that the virus is non-pathogenic, does not require dividing cells, has a broad host range, and is capable of infecting many cell types. Wang and colleagues[29] showed that a single intraportal injection of a recombinant AAV vector encoding canine factor IX complementary DNA under the control of a liver specific enhancer/promoter led to the long term and complete correction of haemophilia in a mouse model. Up to 15–20 mcg/ml of canine factor IX was detected in the plasma of mice for more than five months after intravenous injection of an AAV vector. The activated partial thromboplastin time of treated mice was also corrected.

Simian virus 40 vectors

This vector system utilises a replication deficient variant of SV-40[30] which contains a powerful endogenous promoter. Mice inoculated with this vector showed a high (>90%) transduction efficiency. For example, recombinant SV-40 expressing human bilirubin-UDP-glucuronyltransferase (SV-hBUGT) was delivered to Gunn rats, which are deficient in BUGT, and hence have hyperbilirubinaemia. After sequential inoculations, great and prolonged decreases in bilirubin concentrations were noted, demonstrating the efficacy of transduction.[30]

The advantages of this vector are that it may be produced in high titre, and can be used against a large variety of cells. Its major drawback is its small genome, which limits insert size.

Hybrid viruses

Recently, a novel chimeric adeno-retroviral vector system,[31] a chimeric adenovirus/AAV hybrid vector,[32] and a HSV-1/AAV hybrid[33] have been developed. These hybrid vectors aim to combine the advantages of the component systems, while nullifying the potential drawbacks. For example, the recently developed adenovirus/AAV hybrid[32] has a higher transduction efficiency and greater cloning capacity than either virus alone. In the future, these systems should prove to be useful vehicles for gene therapy.

NON-VIRAL METHODS OF GENE DELIVERY

To overcome the various problems inherent to viral delivery systems, investigators have explored artificial, non-viral gene delivery systems. The most common technique involves the attachment of a therapeutic gene to a carrier. These carriers may be either polymer based cationic carriers (conjugates) or lipid based vectors (liposomes).[34]

In order to improve the specificity of these vectors, the process of receptor mediated endocytosis has been studied[35] using the asialoglycoprotein receptor (AsGPr) which is expressed selectively on hepatocytes. Specific ligands recognised by the AsGPr have been attached to therapeutic genes, usually in combination with a polycation such as polylysine. The DNA–polylysine–ligand complex binds to the receptor, and is taken up by endocytosis. A fraction of the DNA diffuses into the nucleus where it is then expressed. Antisense oligonucleotides may also be targeted specifically to the liver via the AsGPr. Administration of the antisense oligonucleotide blocked the expression of hepatitis B virus in vitro, which consequently reduced the viral burden.[11] Addition of endosomolytic and biocompatible solubilising agents have improved efficiency of expression. None the less, relatively low levels of expression remain a drawback of the system in vivo.

Conclusions

As with all biomedical endeavours, the leap from bench to bedside is a giant one. The last decade of this millennium has seen the science of hepatic genetics come of age. It is true that many challenges remain, but if the past is any indication, they will be countered by the persistence and ingenuity of the investigators in this field. Based on the many advances in the field, there is reason for continued, but guarded optimism for clinical applications of gene therapy for hepatic diseases.

K SHETTY
G Y WU
C H WU

Department of Medicine,
Division of Gastroenterology-Hepatology,
University of Connecticut Health Center,
263 Farmington Avenue
Farmington, CT 06030, USA

Correspondence to: Dr Catherine Wu. email: cwu@nso1.uchc.edu

1 Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat* 1992;1:445–66.
2 Wilson JM, Roy Chowdhury N, Grossman M, *et al.* Temporary amelioration of hyperlipidemia in low density lipoprotein receptor deficient rabbits transplanted with genetically modified hepatocytes. *Proc Natl Acad Sci USA* 1990;87:8437–41.
3 Grossman M, Rader DJ, Muller DW, *et al.* A pilot study of ex vivo gene therapy for homozygous familial hypercholestrolemia. *Nat Med* 1995;1:1148–54.
4 Tada K, Roy-Chowdhury N, Prasad V, *et al.* Long-term amelioration of bilirubin glucuronidation defect in Gunn rats by transplanting genetically modified immortalized autologous hepatocytes. *Cell Transplant* 1998;7:607–16.
5 Raper SE, Wilson JM, Yudkoff M, *et al.* Developing adenoviral-mediated in vivo gene therapy for ornithine transcarbamylase deficiency. *J Inherit Metab Dis* 1998;21(supp 1):119–37.
6 VandenDriessche T, Vanlembrouck V, Goovaerts I, *et al.* Long-term expression of human coagulation factor VIII and correction of hemophilia A after in vivo retroviral gene transfer in factor-VIII deficient mice. *Proc Natl Acad Sci USA* 1999;96:10379–84.
7 Yoon K, Cole-Strauss A, Kmiec EB. Targeted gene correction of episomal DNA in mammalian cells mediated by a chimeric RNA/DNA oligonucleotide. *Proc Natl Acad Sci USA* 1996;93:2071–6.
8 Kren BT, Cole-Strauss A, Kmiec EB, *et al.* Targeted nucleotide exchange in the alkaline phosphatase gene of Huh1-7 cells mediated by a chimeric RNA/DNA oligonucleotide. *Hepatology* 1997;25:1462–8.
9 Kren BT, Bandyopadhyay P, Steer CJ. In vivo site-directed mutagenesis of the factor IX gene by chimeric RNA/DNA oligonucleotides. *Nat Med* 1998;4:285–90.
10 Santoro SW, Joyce GF. A general purpose RNA-cleaving DNA enzyme. *Proc Natl Acad Sci USA* 1997;94:4262–6.
11 Asahina Y, Ito Y, Wu CH, *et al.* DNA ribonucleases that are active against intracellular hepatitis B viral RNA targets. *Hepatology* 1998;28:547–54.
12 Wu GY, Wu CH. Specific inhibition of hepatitis B viral gene expression in vitro by targeted antisense oligonucleotides. *J Biol Chem* 1992;267:12436–9.
13 Offensperger WB, Offensperger S, Walter E, *et al.* In vivo inhibition of duck hepatitis B virus replication and gene expression by phosphorothioate modified antisense oligodeoxynucleotides. *EMBO J* 1993;12:1257–62.
14 Zhang H, Hanecak R, Brown-Driver V, *et al.* Antisense oligonucleotide inhibition of hepatitis C virus (HCV) gene expression in livers of mice infected with an HCV-vaccinia virus recombinant. *Antimicrob Agents Chemother* 1999;43:347–53.
15 Wu CH, Wu GY. Targeted inhibition of hepatitis C virus-directed gene expression in human hepatoma cell lines. *Gastroenterology* 1998;114:1304–12.
16 Huber BE, Richards CA, Austin EA. Virus directed enzyme/prodrugtherapy (VDEPT). Selectively engineering drug sensitivity into tumors. *Ann NY Acad Sci* 1994;716:104–14.
17 Kaneko S, Hallenbeck P, Kotani T, *et al.* Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res* 1995;55:5283–7.
18 Hurford RK Jr, Dranoff G, Mulligan RC, *et al.* Gene therapy of metastatic cancer by in vivo retroviral gene targeting. *Nat Genet* 1995;10:430–5.
19 Alpana GV. Retroviral vectors for liver-directed gene therapy. *Semin Liver Dis* 1999;19:27–37.
20 Patijn GA, Lieber A, Schowalter DB, *et al.* Hepatocyte growth factor induces hepatocyte proliferation in vivo and allows for efficient retroviral-mediated gene transfer in mice. *Hepatology* 1998;28:707–16.
21 Verma IM, Somia N. Gene therapy-promises, problems and prospects. *Nature* 1997;389:239–42.
22 Herz J, Gerard RD. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholestrol clearance in normal mice. *Proc Natl Acad Sci USA* 1993;90:2812–16.
23 Jaffe HA, Danel C, Longenecker G, *et al.* Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. *Nat Genet* 1992;1:372–8.
24 Yang Y, Li Q, Ertl HCJ, *et al.* Cellular and humoral immune responses to viral antigen create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 1995;67:2004–15.
25 Ilan Y, Jona VK, Sengupta K, *et al.* Transient immunosuppression with FK506 permits long-term expression of therapeutic genes introduced into the liver using recombinant adenoviruses. *Hepatology* 1997;26:949–56.
26 Yang Y, Nunes FA, Berencsi K, *et al.* Inactivation of E2a in recombinant adenoviruses limits cellular immunity and improves the prospect of gene therapy of cystic fibrosis. *Nat Genet* 1994;7:362–9.

27 Lee MG, Abina MA, Haddada H, *et al.* The constitutive expression of the immunomodulatory gp 19K protein in E1-, E3-adenoviral vectors strongly reduces the host cytotoxic T cell response against the vector. *Gene Ther* 1995;2:256–62.
28 Amalfitano A, McVie-Wylie AJ, Hu H, *et al.* Systemic correction of the muscle disorder glycogen storage disease type II after hepatic targeting of a modified adenovirus vector encoding human acid-alpha-glucosidase. *Proc Natl Acad Sci USA* 1999;96:8861–6.
29 Wang L, Takabe K, Bidlingmaier SM, *et al.* Sustained correction of bleeding disorder in hemophilia B mice by gene therapy. *Proc Natl Acad Sci USA* 1999;96:3906–10.
30 Strayer DS, Zern MA. Gene delivery to the liver using Simian-Virus 40-derived vectors. *Semin Liver Dis* 1999;19:71–81.
31 Bilbao G, Feng M, Rancourt C, *et al.* Adenoviral/retroviral vector chimers: a novel strategy to achieve high-efficiency stable transduction in vivo. *FASEB J* 1997;11:624–34.
32 Patijn GA, Kay MA. Hepatic gene therapy using adeno-associated virus vectors. *Semin Liver Dis* 1999;19:61–9.
33 Fraefel C, Jacoby DR, Lage C, *et al.* Gene transfer into hepatocytes mediated by helper virus-free HSV/AAV hybrid vectors. *Mol Med* 1997;3:813–25.
34 Farhood H, Gao X, Barsoum J, Huang L. Codelivery to mammalian cells of a transcriptional factor with cis-acting element using cationic liposomes. *Anal Biochem* 1995;225:89–93.
35 Smith RM, Wu GY. Hepatocyte-directed gene delivery by receptor mediated endocytosis. *Semin Liver Dis* 1999;19:83–92.

Regional treatment of hepatic micrometastasis by adenovirus vector-mediated delivery of interleukin-2 and interleukin-12 cDNAs to the hepatic parenchyma

Edward A. Hirschowitz,[1] Hassan A. Naama,[2] Denis Evoy,[1,2] Michael D. Lieberman,[2] John Daly,[2] and Ronald G. Crystal[1]

[1]Division of Pulmonary and Critical Care Medicine and [2]Department of Surgery, New York Hospital-Cornell Medical Center, New York, New York 10021.

We hypothesize that adenovirus (Ad) vector-mediated delivery of the human interleukin-2 (IL-2) cDNA (AdIL2) or the murine IL-12 cDNA heterodimer (AdIL12) would produce high concentrations of cytokines in the local hepatic milieu to induce host responses sufficient to inhibit the growth of experimental colon carcinoma-derived hepatic metastases. Ad vectors administered intravenously, which is a route known to deliver >90% of the vector to the hepatic parenchyma, achieved significant levels of each cytokine locally, with minimal levels in the sera. To examine the therapeutic effect, the AdIL2 and AdIL12 vectors were evaluated in a hepatic metastasis model that was established by injecting 3×10^4 cells from the poorly immunogenic syngeneic C26 colon carcinoma cell line into the right lobe of the livers of BALB/c mice. Animals received AdIL2, AdIL12, or control virus (10^8 plaque-forming units each) intravenously for 2 days after tumor implantation, and tumor growth was compared with naive controls. The AdNull control tumors measured 116 ± 25 mm^2 at 2 weeks. The control virus showed no significant antitumor effect. In marked contrast, both AdIL2 and AdIL12 vectors that were delivered regionally had significant antitumor effects, with AdIL2-treated animals having an average tumor size of 16 ± 8 mm^2; AdIL12-treated tumors measured 6 ± 6 mm^2 ($P < .01$, both compared with control). Both the AdIL2 and AdIL12 vectors provided a significant survival advantage by log-rank analysis ($P < .01$), but only AdIL12 translated into an increase in mean survival from 27 (naive control) to 37 days. To evaluate whether these antitumor effects were T-cell-mediated, splenocytes from AdIL2-treated, AdIL12-treated, and naive control groups were stimulated *in vitro* with γ-irradiated C26 tumor cells for 5 days and tested for C26 tumor cell cytolysis by an *in vitro* cytotoxicity assay. Splenocytes from both AdIL2- and AdIL12-treated animals showed a dose-dependent, T-cell-mediated, specific cytolysis of CT26 cells. AdIL12 and to a lesser extent AdIL2 induced natural killer cell activity, as determined by a dose-dependent increase in lysis of the natural killer-specific target cell YAC-1. Overall, these data suggest that regional Ad-mediated delivery of IL-2 and IL-12 cDNAs may be useful for local tumor control and may warrant further investigation as a potentially useful adjuvant for the treatment of hepatic micrometastasis.

Key words: Adenovirus; interleukin-2; interleukin-12; liver; tumor.

Immunotherapy for metastatic cancer is directed toward stimulating host defenses to recognize and destroy tumor cells.[1] There are two general approaches to immunotherapy: (a) activation of the immune system to identify specific antigens related to tumors and (b) the nonspecific, general activation of immune effector cells.[2] Although the specific activation approach has the elegance of a "surgical strike," it is limited by the requirement of identification of tumor-specific epitopes and by the heterogeneity of tumor antigens in a polyclonal population of tumor cells.[1,2] In contrast, nonspecific activation of the immune system avoids the requirement of knowledge of specific antigens but suffers from the problem of nonspecific systemic toxicity.

In vivo gene therapy is one strategy to limit the toxicities of nonspecific immune-activating mediators by directing genes to a localized area so that the gene product is concentrated in the local milieu, achieving sustained high local concentrations of cytokine, with limited systemic exposure. This approach also offers the theoretical potential for an induction of systemic immunity ("*in situ* vaccination"), similar to approaches using tumor cells transduced with cytokine genes *ex vivo* as tumor vaccines, to achieve immune enhancement in the proximity of tumor antigens.[3,4] Most experimental strategies using *in vivo* gene transfer of cytokine genes target tumor cells directly.[5–19] An alternate approach is to deliver the therapeutic gene to the normal parenchyma of an organ in which the tumor cells are growing.[20] One

Received December 1, 1998; accepted April 3, 1999.

Address correspondence and reprint requests to Dr. Edward A. Hirschowitz, New York Hospital-Cornell Medical Center, 520 East 70th Street, ST505, New York, NY 10021. E-mail address: geneticmedicine@mail.med.cornell.edu

such application might be microscopic hepatic metastases from colon cancer, a logical target because 30% of all individuals with colon cancer will develop liver metastases within 3 years of initial diagnosis.[21,22] The current study evaluates such a regional approach to cytokine gene therapy for the treatment of hepatic micrometastases.

Interleukin-2 (IL-2) and IL-12 are both rational choices for organ-directed *in vivo* gene therapy because of their well-defined immunological profiles and based on the knowledge that the recombinant proteins for each have been used clinically with encouraging results; however, both IL-2 and IL-12 are associated with life-threatening toxicities when administered systemically.[23–30] IL-2 is the primary cytokine responsible for the activation and clonal expansion of $CD8^+$ cytotoxic T lymphocytes (CTLs), which are the primary effector cells that target tumors in an antigen-specific manner.[2] IL-12 functions to activate and clonally expand natural killer (NK) cells, which kill tumors in a major histocompatibility complex (MHC)-independent fashion.[31] IL-12 also has a direct effect on $CD8^+$ lymphocytes[31,32] and induces T helper 1 interferon-γ secretion, with a resulting augmentation of CTL activity, increasing antigen presentation via unregulated MHC class II on the surface of antigen-presenting cells, and in some cases, MHC class I on the surface of tumor cells.[33] Although not used clinically for colon carcinoma, both IL-2 and IL-12 have been shown to be effective against experimental colon-derived tumors.[7,11,34–37]

In the context of these considerations, the present study compares the local antitumor effects and overall survival following adenovirus (Ad)-mediated regional delivery of human IL-2 cDNA and murine IL-12 cDNA to the hepatic parenchyma of mice with established micrometastases derived from a syngeneic colon carcinoma cell line. The data suggest the involvement of a tumor cell-specific cytotoxic T-cell mechanism and a possible role for antigen-independent (NK cell) antitumor mechanisms.

MATERIALS AND METHODS

Ad vectors

The $E1a^-$, partial $E1b^-$, and partial $E3^-$ Ad vector AdIL2 was constructed using the recombination plasmid pCMV.$S2^+$ and the Ad5 pJM17 backbone, as described previously.[38] The AdIL2 vector expression cassette contains the cytomegalovirus immediate early promoter followed by an artificial splice sequence, human IL-2 cDNA (a gift of Bernd Gansbacher, Memorial Sloan-Kettering Cancer Center, New York, NY), and the simian virus 40 polyadenylation sequence. The AdIL12 vector (a gift of Frank Graham, McMaster University, Hamilton, Ontario, Canada) expresses the p35 and p40 subunits of murine IL-12 under the control of the cytomegalovirus promoter;[39] the vector was tested for production of a functional IL-12 protein.[39] The AdCD vector, carrying the *Escherichia coli* cytosine deaminase (CD) cDNA, served as a control virus, because the CD transgene is not therapeutically active unless combined with the prodrug 5-fluorocytosine.[40] All vectors were amplified and titered as described previously.[41]

Cell line

The colon carcinoma cell line C26 is a weakly immunogenic, chemically induced tumor syngeneic to BALB/c mice (National Institutes of Health Division of Cancer Treatment, National Cancer Institute, Frederick, Md).[34] The cell line was maintained in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY) with 10% fetal bovine sera (Biofluids, Rockville, Md), 50 U/mL penicillin G, and 50 mg/mL streptomycin (Biofluids).

AdIL2- and AdIL12-directed production of human IL-2 in vivo

To evaluate the regional liver delivery of cytokines after systemic administration of Ad vectors, AdIL2 (10^8 plaque-forming units (PFU); $n = 3$), AdIL12 (10^8 PFU; $n = 3$), or naive control ($n = 3$) were delivered intravenously (i.v.) to BALB/c mice. At 72 hours, the livers were removed and homogenized in protein lysis buffer (10 mM tris(hydroxymethyl)aminomethane-HCl (pH 7.4), 2% Triton X-100, 0.025% sodium azide, 0.14 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 30 μM leupeptin, 30 μM aprotinin, and 30 μM pepstatin; Sigma, St. Louis, Mo) using a Polytron homogenizer. Total protein was quantified using a BioRad protein assay (Hercules, Calif). IL-2 and IL-12 tissue levels were quantified per milligram of protein using enzyme-linked immunosorbent assays (ELISAs) (human IL-2 from R&D Systems, Minneapolis, Minn; murine IL-12 from Genzyme, Cambridge, Mass). To assess whether there was a significant systemic leak of IL-2 or IL-12 from the liver into the systemic circulation after vector administration to the liver, blood samples taken at the time of sacrifice by right heart puncture were evaluated for levels of IL-2 or IL-12 per milliliter of serum by ELISA, as described above.

Animal model

Intrahepatic tumors were established by injecting 5×10^4 C26 cells suspended in a 30-μL volume through a 30-gauge needle into the left hepatic lobe via laparotomy. With this approach, the development of tumors is consistent, resulting in the development of macroscopic tumors (20–40 mm^2) at 7 days. Tumor area (width $\times$ length, mm^2) was measured using calipers.[42]

The i.v. delivery of Ad vectors leads to infection primarily (>90%) of liver parenchyma;[43,44] thus, i.v. delivery of Ad vectors can be used as a paradigm for "regional therapy" in hepatic tumor models. Ad vectors AdIL2 (10^8 PFU; $n = 5$) or AdIL12 (10^8 PFU; $n = 5$) or the AdCD control virus (10^8 PFU; $n = 5$) were injected in a 100-μL volume into the lateral tail of mice at 2 days after tumor cell implantation. A fourth group of animals receiving no therapy served as an additional control ($n = 5$). Tumor burden was assessed at 14 days. To evaluate survival after therapy, mice receiving tumor implantation were subgrouped to receive i.v. AdIL2 (10^8 PFU; $n = 12$), AdIL12 (10^8 PFU; $n = 12$), the AdCD control virus (10^8 PFU; $n = 11$), or no therapy ($n = 24$) and followed for survival for $\leq$150 days.

Cytotoxic T-cell activity after AdIL12 and AdIL2 delivery in vivo

To determine whether regional AdIL2 or AdIL12 therapy induced specific CTLs, tumors were established as described above; mice were grouped to receive i.v. AdIL2 (10^8 PFU) ($n = 3$), AdIL12 (10^8 PFU) ($n = 3$), or no therapy ($n = 3$) at 2 days after tumor cell implantation as described above. Mice

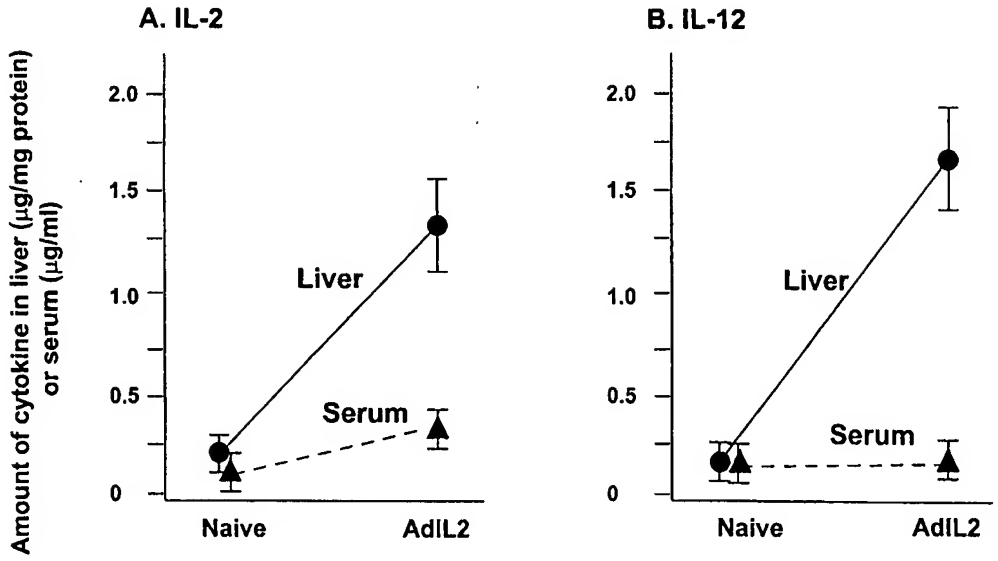


Figure 1. Ad-mediated IL-2 and IL-12 levels in the liver parenchyma and sera after regional delivery of AdIL2 and AdIL12. Ad vectors were delivered i.v., and the levels of each respective cytokine were measured in the liver and sera by ELISA and compared with naive controls. **A:** IL-2. **B:** IL-12. For both cytokines, the liver data are expressed in micrograms per milligrams of protein; serum data are expressed as micrograms per milliliter. Data represent the mean ± SE.

were sacrificed at 12 days after vector delivery, and splenic mononuclear cells were isolated as described previously.[45] Briefly, spleens harvested from each group were minced and ground, sheared with a 19-gauge needle, and passed through a 200-μm mesh to remove fibrous tissue. Live lymphocytes were separated from dead cells and red blood cells using Ficoll-Paque (Pharmacia, Piscataway, NJ) density separation, washed, and resuspended in Dulbecco's modified Eagle's medium. Prepared splenocytes were stimulated *in vitro* for 5 days with γ-irradiated (50 Gy) C26 cells. The tumor-specific CTL activity in the cultured, primed splenocytes from each group was quantified using a lactate dehydrogenase (LDH) release cytotoxicity assay (Promega, Madison, Wis) against C26 target cells by coincubating effector and target cells at various ratios (6:1–100:1) for 4 hours at 37°C in serum-free medium. LDH was assayed in the supernatant by optical density (OD) at 490 nm,[46] and target cell lysis was calculated as follows: 100 × ([OD of sample − OD of spontaneous release of LDH from target cells − OD of spontaneous release of LDH from effector cells]/[OD of maximal release from target cells − OD of spontaneous release from target cells]).[46]

NK activity

Splenocytes from mice at 7 days after i.v. injection of AdIL12 (10^8 PFU, $n = 3$), AdIL2 (10^8 PFU, $n = 3$), AdCD control virus (10^8 PFU, $n = 3$), or naive control ($n = 3$) were evaluated for cytolysis of YAC-1 murine hybridoma cells (American Type Culture Collection, Manassas, Va), which serve as a specific target for NK cells.[47] Splenocytes were harvested from the animals in each group and pooled; mononuclear cells were isolated as described above. Fresh, pooled splenocytes from each group were incubated with 10^4 YAC-1 cells in increasing effector to target (E:T) cell ratios (0:1, 10:1, and 100:1) for 4 hours at 37°C in serum-free medium. LDH was assayed in the supernatant by OD, and target cell lysis was calculated as described for the CTL assay.

Statistical analysis

Data are presented as mean ± SEM. All comparisons were made using an unpaired two-tailed Student's *t* test. Survival advantage was measured using the log-rank test.

RESULTS

Ad-mediated liver production of IL-2 and IL-12

To evaluate the production of IL-2 and IL-12 within the hepatic parenchyma following a single dose, the systemic delivery of AdIL2 or AdIL12, IL-2, and IL-12 was quantified at 72 hours in the liver and compared with naive controls. The livers of AdIL12-treated mice contained large amounts of IL-2 ($P < .01$ compared with naive controls; Fig 1); the livers of AdIL12-treated mice contained large amounts of IL-12 ($P < .01$ compared with naive controls). In contrast, the AdIL2-treated animals had only a minor increase in sera IL-2 levels, which were not significantly different from the controls ($P = .5$). Sera from AdIL12-treated animals contained IL-12 levels similar to the naive controls ($P > .1$). These observations demonstrate that there is local production of the therapeutic cytokines within the liver after administration of the relevant Ad vector, although the pharmacokinetic levels of tissue are undoubtably complex.

Antitumor activity of regionally delivered AdIL2 and AdIL12

Syngeneic CT26 colon carcinoma cells grow in the liver in a reproducible manner, with tumors of 100–130 mm^2 growing in 14 days (Fig 2). The tumors grew to a

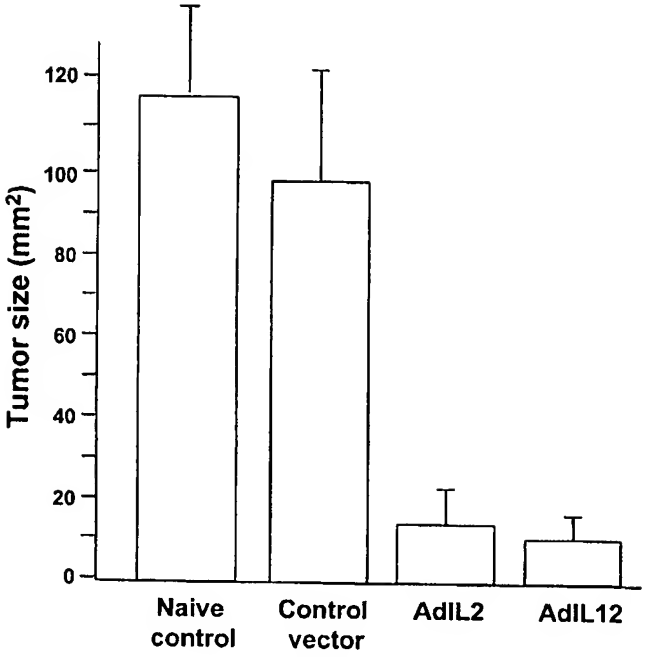


Figure 2. Therapeutic effects of Ad-mediated regional expression of IL-2 and IL-12. Hepatic micrometastases of CT26 syngeneic colon carcinoma cells were established in BALB/c mice, and mice were subgrouped to receive AdIL2, AdIL12, control virus, or no therapy at 2 days after tumor cell implantation. Tumor size was measured in two dimensions, and data are expressed as tumor area (mm^2) ± SE at 14 days.

comparable size in mice receiving the control vector ($P > .1$). In contrast, mice receiving AdIL2 had a marked clearance in liver tumor burden ($P < .01$), as did mice receiving AdIL12 ($P < .01$). There was no difference in the tumor burden of the animals receiving AdIL2 or AdIL12 ($P > .1$).

Median survival

To evaluate whether the reduction in tumor burden at 2 weeks translated into a survival advantage, mice were grouped as described above and monitored until death (Fig 3). The median survival of naive mice (24 days) was similar to that for mice receiving the control vector (27 days). The median survival of the mice receiving AdIL2 was 28 days. AdIL12 increased the mean survival to 38 days. Although the mean increase in survival after AdIL2 therapy was marginal, both AdIL2 and AdIL12 therapies provided a statistically significant survival advantage as measured by log-rank analysis ($P < .01$ for both AdIL2 and AdIL12 compared with the control). There was one apparent cure (≥150 days) in the AdIL2-treated group.

Cytotoxic T-cell activity after AdIL12 and AdIL2 delivery

To assess whether regional delivery of AdIL2 and AdIL12 to liver-bearing micrometastases was capable of inducing specific T-cell-mediated immunity, splenocytes from AdIL2-treated animals, AdIL12-treated animals, and "untreated tumor-bearing" controls were tested for C26 tumor cell-specific cytolysis. Splenocytes from both AdIL2- and AdIL12-treated animals showed a dose-dependent T-cell-mediated response, indicating that both vectors are capable of inducing T-cell immunity when delivered *in vivo* in the milieu of micrometastatic disease (Fig 4).

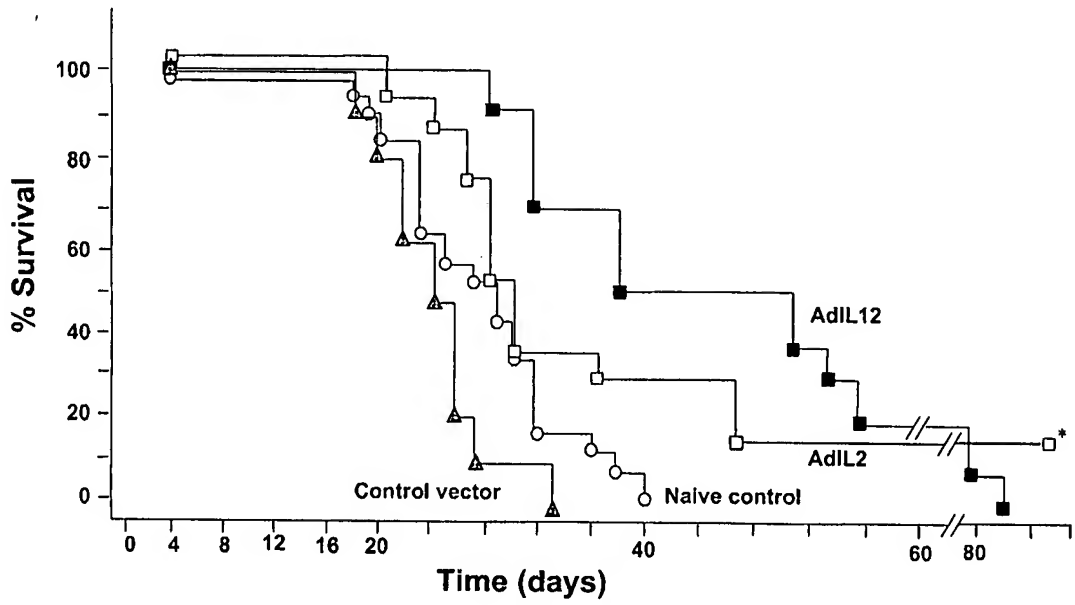


Figure 3. Survival of mice after Ad-mediated regional expression of IL-2 and IL-12. Mice received therapeutic vectors i.v. at 2 days after tumor cell implantation and were followed for survival. Data are expressed as the percentage of survival over time. The groups included AdIL2, AdIL12, the AdCD control virus, and naive controls.

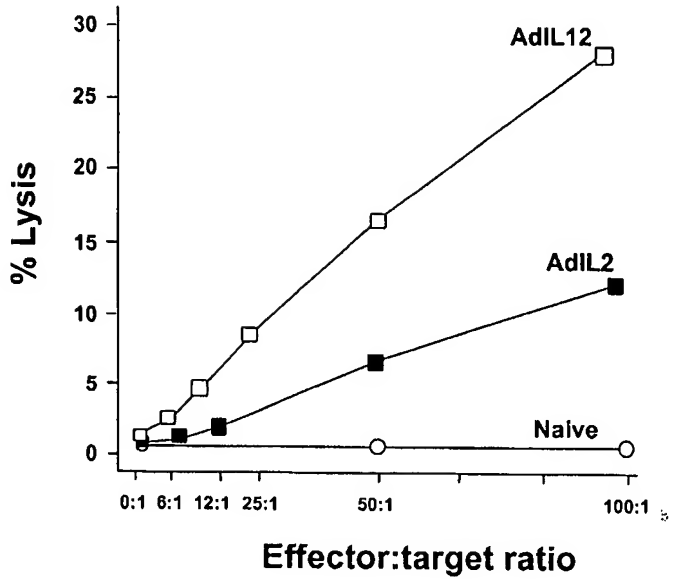


Figure 4. Assessment of tumor-specific CTLs after treatment of hepatic micrometastases. Tumor-bearing mice were treated with AdIL2 or AdIL12 at 2 days after tumor cell implantation; animals receiving no therapy served as a control. Splenocytes were isolated at 12 days after vector delivery and stimulated *in vitro* with γ-irradiated C26 cells for 5 days. The cultured splenocytes were evaluated for specific lysis against C26 tumor cells in a cytotoxicity assay. Each datapoint represents the percentage of specific lysis of pooled lymphocytes from three animals. Data are expressed as the percentage of specific lysis at various E:T cell ratios.

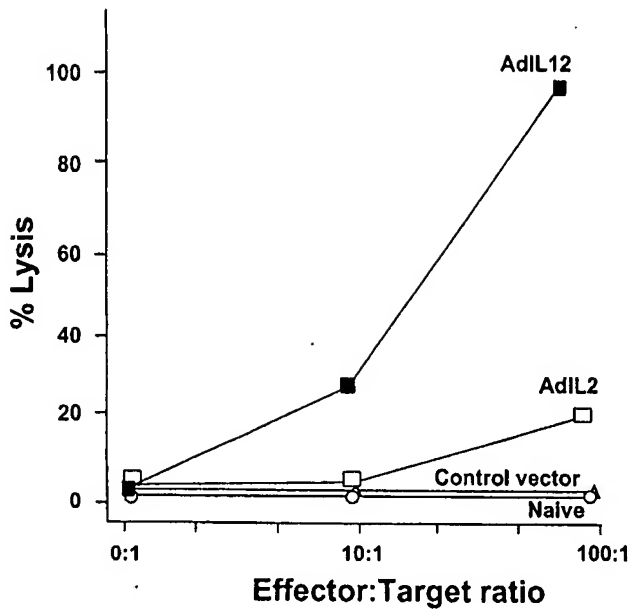


Figure 5. Assessment of NK activity after administration of AdIL2 and AdIL12. Non-tumor-bearing mice received a single dose of AdIL12, AdIL2, or control virus or received no therapy. On day 7, splenocytes were assessed for their ability to lyse the NK-specific target cell YAC-1 using a cytotoxicity assay. Data are expressed as the percentage of specific lysis at various E:T cell ratios. Each datapoint represents the percentage of specific lysis of pooled lymphocytes from three animals.

NK activity after AdIL2 and AdIL12 delivery

To assess the ability of AdIL2 and AdIL12 to induce NK cell activity *in vivo*, naive (non-tumor-bearing) splenocytes from mice that had received a single dose of AdIL2, AdIL12, or control virus were evaluated for the percentage of specific lysis of the NK-specific target cell YAC-1 (Fig 5). AdIL12 delivery induced marked NK cell activity. AdIL2 also induced NK activity, but to a much lesser extent.

DISCUSSION

Based on the well-established antitumor profile of IL-2 and IL-12 cytokine therapy,[2,28] the current study explores the potential of delivering the cDNAs of these two cytokines to the hepatic parenchyma to act as a regional therapy for hepatic metastases. Capitalizing on the fact that i.v. delivery of Ad vectors delivers >90% of the Ad genome to the liver,[43,44] the IL-2 and IL-12 cDNAs were delivered to the livers of BALB/c mice using an Ad vector as an experimental regional therapy for established hepatic micrometastases derived from the syngeneic colon cancer cell line C26. The data show that i.v. delivery of AdIL2 and AdIL12 yields significant levels of each cytokine within the hepatic parenchyma, with no concomitant increase in sera levels. Therapeutically, both vectors are capable of reducing tumor burden in the liver by a significant margin. In addition, both therapies yielded a significant survival advantage by log-rank analysis, although only AdIL12 treatment translated into a increase in mean survival. The mechanisms of antitumor effect of both vectors are associated with the generation of tumor-specific cytotoxic T cells as well as NK cell activity. The relative increase in cell-mediated immunity (both T-cell and NK activity) associated with AdIL12-treated animals compared with AdIL2-treated animals in these *in vitro* assays suggests a therapeutic advantage of AdIL12 therapy, although these *in vitro* differences were not reflected in the relative suppression of tumor growth. However, these data could help to explain the relative increase in mean survival after AdIL12 therapy compared with AdIL2 therapy.

In vivo *Ad-mediated cytokine regional expression in hepatic parenchyma*

Cytokine therapy for cancer is intended to direct host immunity to tumor cells.[1] Serial administrations of recombinant cytokines have been used experimentally and clinically with some therapeutic success, but also with unwanted systemic toxicity resulting from supranormal circulating levels of cytokines following high dose bolus delivery or the narrow therapeutic index of sustained infusion.[23–25,28–30,43,48,49] Gene therapy is a strategy in which cytokines can be produced at sustained doses in a paracrine fashion and localized to the site organ of the

tumor.[1,3–19,50–54] *Ex vivo* gene therapy vaccine strategies use cytokine gene-modified tumor (or normal) cells to enhance host immunity at the site of the tumor cells and at tumor foci at distant sites.[50–54] *In vivo* strategies target tumor cells *in situ*, without having to modify cells *ex vivo*. *In vivo* gene therapy has been carried out using IL-2 and IL-12 cDNAs in experimental tumor models.[5–19] This approach strives to enhance the immune recognition of tumor cells at the site of the primary tumor and may induce systemic antitumor immunity that is effective at distant sites.[6,10]

The current study evaluates the use of *in vivo* cytokine gene therapy for microscopic metastases localized within a single organ. However, the approach is different from that used in prior studies.[5–19] Rather than directing the gene therapy to the tumor mass, the gene therapy is directed toward the organ overall. Regionally directed *in vivo* gene therapy offers an approach to treat microscopic disease without having to target tumor cells directly. Experimentally, regional delivery to the hepatic parenchyma of small animals using Ad vectors can be accomplished by intravenous, intraportal, or intrahepatic arterial injection of vector.[42–44,55] Importantly, there is minimal risk from the Ad vector *per se*, with systemic (i.v.) administration of $\leq 10^9$ PFU Ad vectors to mice inducing only mild, self-limited inflammation within the hepatic parenchyma.[20] Although it is possible that the inflammatory mediators associated with Ad vector infection *per se* (not measured in these studies) might contribute to the therapeutic effects seen with AdIL2 and AdIL12 administration, Ad vector-mediated inflammation is unlikely to play a significant role; Ad vector alone (at 10^8 PFU) had no independent therapeutic effect. Comparisons of the efficacy and toxicity of this therapy to that of systemically administered cytokines remain to be evaluated. Another strategy of locally administered gene therapy to the normal liver parenchyma includes the hepatic delivery of the gene for the prodrug CD.[20,42]

Survival and induction of systemic immunity

The potential for the induction of systemic antitumor immunity after *in vivo* regional therapy of AdIL2 or AdIL12 for micrometastases is suggested by the finding of specific CTLs directed against parental tumor cells *in vitro*. The induction of NK cells by AdIL12 and to a lesser extent AdIL2 that was observed after i.v. delivery of these vectors may offer systemic antitumor immunity in an MHC class I-independent fashion. However, it can be seen through survival experiments that *in vivo* antitumor immunity after a single dose of these Ad vectors is insufficient to effect a cure in this model. Although both therapies show a significant survival advantage by log-rank analysis and a similar reduction of tumor burden at 2 weeks, it is unclear why there is a discrepancy between the mean survival advantage seen in AdIL12-treated animals and the advantage seen in AdIL2-treated animals. One explanation is that the measurement of tumor area at 2 weeks may not reflect actual differences in viable cell number within the hepatic tumors; fewer viable cells present within the AdIL12-treated tumors would lead to a significant delay of the growth curve of these tumors, and thus mortality, when compared with AdIL2-treated mice and controls. Alternatively, IL-12 expression may induce or potentiate lasting immunity through its effects on antigen-presenting cells and T helper 1 cells, which are important in maintaining immune memory.[2] In contrast, IL-2, whose antitumor effects are thought to result from direct stimulation of effector cells, acts to bypass rather than augment these mechanisms of cellular immunity.[34,37] A combination of these two cytokines is logical and has been shown to augment the antitumor effects of either cytokine alone.[56,57] This combination may offer further significant antitumor immunity through increases in effector cell number and function and remains to be explored using a regional *in vivo* approach. The current study nonetheless offers an additional approach for local control of tumors, with the potential for systemic immunity; further benefit may be gained experimentally with less aggressive and more immunogenic tumor cell lines and potentially with primary tumors in a clinical setting.

ACKNOWLEDGMENTS

We thank N. Mohamed for help in preparing this manuscript. These studies were supported in part by the National Institutes of Health/National Heart, Lung, and Blood Institute (R01 CA 75192 and R21 CA 75153), the Will Rogers Memorial Fund (Los Angeles, Calif), and GenVec, Inc. (Bethesda, Md).

REFERENCES

1. Pardoll DM. Paracrine cytokine adjuvants in cancer immunotherapy. *Annu Rev Immunol.* 1995;13:399–415.
2. Lanzavecchia A. Identifying strategies for immune intervention. *Science.* 1993;260:937–944.
3. Tepper RI, Mule JJ. Experimental and clinical studies of cytokine gene-modified tumor cells. *Hum Gene Ther.* 1994;5:153–164.
4. Gilboa E. Immunotherapy of cancer with genetically modified tumor vaccines. *Semin Oncol.* 1996;23:101–107.
5. Haddada H, Ragot T, Cordier L, et al. Adenoviral interleukin-2 gene transfer into p815 tumor cells abrogates tumorigenicity and induces antitumoral immunity in mice. *Hum Gene Ther.* 1993;4:703–711.
6. Addison CL, Braciak T, Ralston R, et al. Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model. *Proc Natl Acad Sci USA.* 1995;92:8522–8526.
7. Chen SH, Chen XH, Wang Y, et al. Combination gene therapy for liver metastasis of colon carcinoma in vivo. *Proc Natl Acad Sci USA.* 1995;92:2577–2581.
8. Cordier L, Duffour MT, Sabourin JC, et al. Complete recovery of mice from a pre-established tumor by direct intratumoral delivery of an adenovirus vector harboring the murine IL-2 gene. *Gene Ther.* 1995;2:16–21.
9. Hurford RK Jr, Dranoff G, Mulligan RC, et al. Gene therapy of metastatic cancer by in vivo retroviral gene targeting. *Nat Genet.* 1995;10:430–435.
10. Bramson JL, Hitt M, Addison CL, et al. Direct intratu-

moral injection of an adenovirus expressing interleukin-12 induces regression and long-lasting immunity that is associated with highly localized expression of interleukin-12. *Hum Gene Ther.* 1996;7:1995–2002.
11. Caruso M, Pham-Nguyen K, Kwong YL, et al. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc Natl Acad Sci USA.* 1996;93:11302–11306.
12. Huang H, Chen SH, Kosai K, et al. Gene therapy for hepatocellular carcinoma: long-term remission of primary and metastatic tumors in mice by interleukin-2 gene therapy in vivo. *Gene Ther.* 1996;3:980–987.
13. Qin H, Chatterjee SK. Recombinant vaccinia expressing interleukin-2 for cancer gene therapy. *Cancer Gene Ther.* 1996;3:163–167.
14. Toloza EM, Hunt K, Swisher S, et al. In vivo cancer gene therapy with a recombinant interleukin-2 adenovirus vector. *Cancer Gene Ther.* 1996;3:11–17.
15. Bui LA, Butterfield LH, Kim JY, et al. In vivo therapy of hepatocellular carcinoma with a tumor-specific adenoviral vector expressing interleukin-2. *Hum Gene Ther.* 1997;8: 2173–2182.
16. Chen L, Chen D, Block E, et al. Eradication of murine bladder carcinoma by intratumor injection of a bicistronic adenoviral vector carrying cDNAs for the IL-12 heterodimer and its inhibition by the IL-12 p40 subunit homodimer. *J Immunol.* 1997;159:351–359.
17. Levraud JP, Duffour MT, Cordier L, et al. IL-2 gene delivery within an established murine tumor causes its regression without proliferation of preexisting antitumor-specific CTLs. *J Immunol.* 1997;158:3335–3343.
18. Pützer BM, Hitt M, Muller WJ, et al. Interleukin 12 and B7 costimulatory molecule expressed by an adenovirus vector act synergistically to facilitate tumor regression. *Proc Natl Acad Sci USA.* 1997;94:10889–10894.
19. Pützer BM, Bramson JL, Addison CL, et al. Combination therapy with interleukin-2 and wild-type p53 expressed by adenoviral vectors potentiates tumor regression in a murine model of breast cancer. *Hum Gene Ther.* 1998;9:707–718.
20. Ohwada A, Hirschowitz EA, Crystal RG. Regional delivery of an adenovirus vector containing the *Escherichia coli* cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum Gene Ther.* 1996;7:1567–1576.
21. Willett CG, Tepper JE, Cohen AM, et al. Failure patterns following curative resection of colonic carcinoma. *Ann Surg.* 1984;200:685–690.
22. Ballantyne GH, Quin J. Surgical treatment of liver metastases in patients with colorectal cancer. *Cancer.* 1993;71: 4252–4266.
23. Rosenberg SA, Lotze MT, Muul LM, et al. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. *N Engl J Med.* 1987;316:889–897.
24. Lee RE, Lotze MT, Skibber JM, et al. Cardiorespiratory effects of immunotherapy with interleukin-2. *J Clin Oncol.* 1989;7:7–20.
25. Margolin KA, Rayner AA, Hawkins MJ, et al. Interleukin-2 and lymphokine-activated killer cell therapy of solid tumors: analysis of toxicity and management guidelines. *J Clin Oncol.* 1989;7:486–498.
26. Dalgleish AG. The role of IL-2 in gene therapy. *Gene Ther.* 1994;1:83–87.
27. Orange JS, Salazar-Mather TP, Opal SM, et al. Mechanism of interleukin 12-mediated toxicities during experimental viral infections: role of tumor necrosis factor and glucocorticoids. *J Exp Med.* 1995;181:901–914.
28. Brunda MJ, Luistro L, Rumennik L, et al. Antitumor activity of interleukin 12 in preclinical models. *Cancer Chemother Pharmacol.* 1996;38:S16–S21.
29. Lamont AG, Adorini L. IL-12: a key cytokine in immune regulation. *Immunol Today.* 1996;17:214–217.
30. Leonard JP, Sherman ML, Fisher GL, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-γ production. *Blood.* 1997; 90:2541–2548.
31. Zitvogel L, Lotze MT. Role of interleukin-12 (IL12) as an anti-tumour agent: experimental biology and clinical application. *Res Immunol.* 1995;146:628–638.
32. Trinchieri G, Scott P. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions. *Res Immunol.* 1995;146:423–431.
33. Fujiwara H, Zou JP, Herrmann S, et al. A sequence of cellular and molecular events involved in IL12-induced tumour regression. *Res Immunol.* 1995;146:638–644.
34. Fearon ER, Pardoll DM, Itaya T, et al. Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. *Cell.* 1990;60: 397–403.
35. Colombo MP, Vagliani M, Spreafico F, et al. Amount of interleukin 12 available at the tumor site is critical for tumor regression. *Cancer Res.* 1996;56:2531–2534.
36. Rao JB, Chamberlain RS, Bronte V, et al. IL-12 is an effective adjuvant to recombinant vaccinia virus-based tumor vaccines: enhancement by simultaneous B7-1 expression. *J Immunol.* 1996;156:3357–3365.
37. Rodolfo M, Zilocchi C, Melani C, et al. Immunotherapy of experimental metastases by vaccination with IL gene-transduced adenocarcinoma cells sharing tumor-associated antigens: comparison between IL-12 and IL-2 gene-transduced tumor cell vaccines. *J Immunol.* 1996;157: 5536–5542.
38. Hirschowitz EA, Leonard S, Song W, et al. Adenovirus-mediated expression of melanoma antigen gp75 as immunotherapy for metastatic melanoma. *Gene Ther.* 1998;5: 1–9.
39. Bramson J, Hitt M, Gallichan WS, et al. Construction of a double recombinant adenovirus vector expressing a heterodimeric cytokine: in vitro and in vivo production of biologically active interleukin-12. *Hum Gene Ther.* 1996;7: 333–342.
40. Hirschowitz EA, Ohwada A, Pascal WR, et al. In vivo adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther.* 1995;6:1055–1063.
41. Rosenfeld MA, Yoshimura K, Trapnell BC, et al. In vivo transfer of the human cystic fibrosis transmembrane conductance regulator gene to the airway epithelium. *Cell.* 1992;68:143–155.
42. Topf N, Worgall S, Hackett NR, et al. Regional "prodrug" gene therapy: intravenous administration of an adenoviral vector expressing the *E. coli* cytosine deaminase gene and systemic administration of 5-fluorocytosine suppresses growth of hepatic metastasis of colon carcinoma. *Gene Ther.* 1998;5:507–513.
43. Herz J, Gerard RD. Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice. *Proc Natl Acad Sci USA.* 1993;90:2812–2816.

44. Worgall S, Wolff G, Falck-Pedersen E, et al. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum Gene Ther.* 1997;8:37–44.
45. Song W, Kong HL, Traktman P, et al. Cytotoxic T lymphocyte responses to proteins encoded by heterologous transgenes transferred in vivo by adenoviral vectors. *Hum Gene Ther.* 1997;8:1207–1217.
46. Decker T, Lohmann-Matthes ML. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods.* 1988; 115:61–69.
47. Gately MK, Warrier RR, Honasoge S, et al. Administration of recombinant IL-12 to normal mice enhances cytolytic lymphocyte activity and induces production of IFN-γ in vivo. *Int Immunol.* 1994;6:157–167.
48. Ardizzoni A, Bonavia M, Viale M, et al. Biologic and clinical effects of continuous infusion interleukin-2 in patients with non-small cell lung cancer. *Cancer.* 1994;73: 1353–1360.
49. Legha SS, Gianan MA, Plager C, et al. Evaluation of interleukin-2 administered by continuous infusion in patients with metastatic melanoma. *Cancer.* 1996;77:89–96.
50. Elder EM, Lotze MT, Whiteside TL. Successful culture and selection of cytokine gene-modified human dermal fibroblasts for the biologic therapy of patients with cancer. *Hum Gene Ther.* 1996;7:479–487.
51. Lotze MT, Rubin JT, Carty S, et al. Gene therapy of cancer: a pilot study of IL-4-gene-modified fibroblasts admixed with autologous tumor to elicit an immune response. *Hum Gene Ther.* 1994;5:41–55.
52. Sobol RE, Royston I, Fakhrai H, et al. Injection of colon carcinoma patients with autologous irradiated tumor cells and fibroblasts genetically modified to secrete interleukin-2 (IL-2): a phase I study. *Hum Gene Ther.* 1995;6:195–204.
53. Tahara H, Zeh HJ III, Storkus WJ, et al. Fibroblasts genetically engineered to secrete interleukin 12 can suppress tumor growth and induce antitumor immunity to a murine melanoma in vivo. *Cancer Res.* 1994;54:182–189.
54. Zitvogel L, Tahara H, Robbins PD, et al. Cancer immunotherapy of established tumors with IL-12: effective delivery by genetically engineered fibroblasts. *J Immunol.* 1995;155:1393–1403.
55. Jaffe HA, Danel C, Longenecker G, et al. Adenovirus-mediated in vivo gene transfer and expression in normal rat liver. *Nat Genet.* 1992;1:372–378.
56. Pappo I, Tahara H, Robbins PD, et al. Administration of systemic or local interleukin-2 enhances the anti-tumor effects of interleukin-12 gene therapy. *J Surg Res.* 1995;58: 218–226.
57. Vagliani M, Rodolfo M, Cavallo F, et al. Interleukin 12 potentiates the curative effect of a vaccine based on interleukin 2-transduced tumor cells. *Cancer Res.* 1996; 56:467–470.